DEVELOPMENTAL REGULATION OF PLACENTAL AND FETAL AMINO ACID TRANSPORT: THE EFFECTS OF LOW-PROTEIN DIET-INDUCED INTRAUTERINE GROWTH RETARDATION

By

MARC SHANE MALANDRO

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1995

ACKNOWLEDGMENTS

I would like to thank the members of my supervisory committee: Drs. Brian Cain, Susan Frost, Mike Kilberg, Harry Nick, Don Novak, and Bruce Stevens. A special word of thanks goes to my co-mentors Mike Kilberg and Don Novak. Without them, this study would not have been possible. Thanks also go to Mark Beveridge and Dr. Jamie Matthews for valuable technical support. I would also like to acknowledge Drs. Roney Laine and Bob McMahon. They both have been and will continue to be good friends and valuable resources. Most of all, I would like to thank my wife, Jennifer and daughter, J. Nicole for support and love throughout this study.

TABLE OF CONTENTS

ACKNOWLEDGMENTS....................................ii

LIST OF TABLES.....................................v

LIST OF FIGURES....................................vi

ABSTRACT...viii

CHAPTER 1 INTRODUCTION.............................1

- Overview of Mammalian Amino Acid Transport.........1
- Introduction to the Placenta.......................8
- Molecular Biology of Charged Mammalian Amino Acid Transporters.............................15

CHAPTER 2 MATERIALS AND METHODS....................57

- Materials..57
- Methods..58

CHAPTER 3 DEVELOPMENTAL REGULATION OF CATIONIC AMINO ACID TRANSPORT IN RAT PLACENTA....................66

- Introduction.......................................66
- Results..70
- Discussion...87

CHAPTER 4 DEVELOPMENTAL REGULATION OF PLACENTAL AND FETAL LIVER ANIONIC AMINO ACID TRANSPORT AND THE CHARACTERIZATION OF A RAT GLUTAMATE TRANSPORTER...93

- Introduction.......................................93
- Methods..99
- Results..103
- Discussion...134

CHAPTER 5 THE EFFECTS OF MATERNAL PROTEIN RESTRICTION ON PLACENTAL AND FETAL LIVER AMINO ACID TRANSPORT IN THE RAT..............................138

- Introduction.......................................138
- Results..141
- Discussion...159

CHAPTER 6 DEVELOPMENT OF A STABLE MAMMALIAN EXPRESSION SYSTEM FOR THE CLONING OF AMINO ACID TRANSPORTERS....................................165

Introduction..165
Methods...168
Results...173
Discussion..183

CHAPTER 7 CONCLUSIONS AND FUTURE DIRECTIONS..............187

LIST OF REFERENCES.....................................190

BIOGRAPHICAL SKETCH.....................................207

LIST OF TABLES

Table page

3-1. Characterization of apical and basal membrane vesicles from rat placentas of 14 or 20 days gestation.....71

3-2. Inhibition of Na^+-dependent (System $B^{0,+}$) arginine uptake in microvillous membrane vesicles from placentas of 20 days gestation.....78

3-3. Inhibition of Na^+-independent, leucine-sensitive arginine uptake in apical and basal membrane vesicles from placentas of 20 days gestation.....79

3-4. Inhibition of Na^+-independent, leucine-insensitive (System y^+) arginine uptake in apical and basal membrane vesicles form placentas of 20 days gestation.....81

4-1. Transient and stable expression of rat EAAC1.....109

5-1. Maternal weight gain, fetal weight, placental weight, and litter size for control and low-protein animals representing three independent experimental trials.....143

5-2. Maternal serum amino acid levels from control and low-protein fed dams.....145

5-3. Transport of MeAIB (System A) in placental apical and basal membrane vesicles from control and low-protein diet-fed animals.....151

5-4. Na^+-dependent transport of glutamate (System X^-_{AG}) in placental basal membrane vesicles from control and low-protein diet-fed animals.....153

LIST OF FIGURES

Figure page

3-1. Time-course of [^{3}H]-arginine uptake in basal or apical membrane vesicles from placentas of 20 days gestation....74

3-2. Inhibition of Na^{+}-dependent [^{3}H]-arginine uptake with increasing concentrations of leucine in placentas of 20 days gestation....76

3-3. Ontogeny of cationic amino acid transport systems in microvillous and basal membrane vesicles....84

3-4. Northern analysis of System y^{+} mRNA from placentas of increasing gestational age....86

4-1. Deduced amino acid sequence of rat EAAC1 cDNA....105

4-2. Tissue distribution or rat EAAC1 mRNA....107

4-3. Synthesis and purification of MBP-EAAC1 fusion protein....111

4-4. Stable expression of rat EAAC1 in human 293c18 cells....114

4-5. Immunoblot analysis of over-expressed rat EAAC1....116

4-6. Time-course of [^{3}H]-glutamate uptake in basal or apical membrane vesicles from placentas of 14 or 20 days gestation....119

4-7. Reverse transcriptase PCR detection of glutamate transporters in placentas at 14 and 20 days gestation....121

4-8. Northern analysis of EAAC1 mRNA from placentas of 14 or 20 days gestation....124

4-9. Immunoblot analysis of EAAC1 protein in apical and basal membrane vesicles from placentas of 20 days gestation....126

4-10. Immunoblot analysis of EAAC1 from placentas of 14 and 20 days gestation....129

4-11.Uptake of glutamate in fetal, newborn, and adult hepatocytes....................................131

4-12. Reverse Transcriptase PCR detection of glutamate transporters in fetal hepatocytes.................133

5-1. Immunoblot analysis of Ca^{2+}ATPase in homogenate, apical, and basal membrane vesicles from control and low-protein fed animals........................149

5-2. Na^+-dependent and Na^+-independent cationic amino acid uptake in apical and basal membrane vesicles from control and low-protein fed animals...........156

5-3. Northern analysis of placental System y^+ (CAT1) and System X^-_{AG} (EAAC1) mRNA from control and low-protein fed animals.............................158

6-1. Diagram and vector map of λDR2/pDR2................171

6-2. Schematic diagram of library screening protocol....176

6-3. Crystal violet stain and autoradiogram of a representative screening with EAAC1-transfected 293c18 cells..179

6-4. PCR detection of EAAC1 from a positive (pDR/EAAC1) 293c18 colony......................................181

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

DEVELOPMENTAL REGULATION OF PLACENTAL AND FETAL AMINO ACID TRANSPORT: THE EFFECTS OF LOW-PROTEIN DIET-INDUCED INTRAUTERINE GROWTH RETARDATION

By

Marc Shane Malandro

December, 1995

Chairman: Dr. Michael S. Kilberg
Major Department: Biochemistry and Molecular Biology

Gestational regulation of the placental transfer of amino acids from maternal to fetal circulations is essential for the proper development of the fetus. Both the cationic and anionic amino acid transport systems of the apical (maternal-facing) and basal (fetal-facing) membranes of the rat placental syncytiotrophoblast were examined. Inhibition analysis documented the presence of three kinetically distinct cationic amino acid transport mechanisms: a single Na^+-dependent mechanism in the apical membrane which increased in activity from 14 to 20 days gestation but was absent from the basal membrane throughout the entire gestational period (System $B^{o,+}$); and two Na^+-independent transport systems in both membrane domains, one completely inhibited by leucine which increased in activity in both the microvillous and basal membrane domains, and the other leucine insensitive which remained fairly constant in the basal membrane and increased throughout gestation in the microvillous membrane (System y^+). Anionic amino acid uptake is mediated by exclusively Na^+-dependent systems on both the apical and basal membranes. Northern analysis with cDNA probes to anionic (EAAC1) and cationic (CAT1) amino acid transporters identified specific mRNA species which increased with increasing gestational age. Concomitant regulation of EAAC1 mRNA, protein, and transport activity was also observed. Induction of intrauterine growth retardation by low-protein diet resulted in the decreased transport of amino acid in both the apical and basal membranes. As several key amino acid transporters have yet to be cloned, a novel mammalian cell expression system was also developed and characterized with a known (EAAC1) transporter.

CHAPTER 1
INTRODUCTION

Overview of Mammalian Amino Acid Transport

The scope of the function of amino acids lies well beyond the role of these molecules as biosynthetic precursors to proteins. These compounds function as carriers of nitrogen and carbon atoms, as metabolic fuels, as neurotransmitters, and as sensitive regulators of cell osmolarity. These molecules must be supplied to or made in the cells to maintain intracellular concentrations of both essential and nonessential amino acids.

Nonessential amino acids can be supplied to cells in a variety of ways. First, these compounds can be synthesized *de novo* in the cell from precursor molecules including α-keto acids and other amino acids. Nonessential amino acids can be synthesized in other cell types and delivered to the cells in the extracellular medium by a process of "interorgan nutrition" (Christensen, 1982). Degradation of existing cellular proteins can also supply these nutrients. On the other hand, essential amino acids must be supplied to the cells only by degradation of intracellular proteins or by delivery of these nutrients to the cells from exogenous sources. This delivery of amino acids from the diet as protein digestion products was described as early as 1913 (Van Slyke and Meyer, 1913). Clearly, the availability of amino acids or amino acid precursors to the extracellular face of cells is not sufficient for the intracellular metabolism of these compounds. These molecules must traverse the plasma membrane which is highly impermeable to charged and polar solutes. As such, mechanisms for delivery of amino acids are not only absolutely necessary for cellular function, but also, the regulation of this delivery may serve as an additional level of control of cellular metabolism.

Introduction to Transport

The term transport refers to the transfer of a solute across the plasma membrane, and the protein(s) that mediate this event is referred to as a transporter. The transport process itself can be visualized in four arbitrarily defined steps: 1) the binding of the solute molecule to the transporter on the extracellular face of the cell; 2) the conformational change in the transport protein resulting in the movement of the solute across the membrane; 3) the release of the solute molecule on the intracellular side of the membrane; 4) the re-orientation of the transporter such that the solute binding site faces the extracellular medium.

Kinetically, as the steps involved in membrane transport are mediated by proteins, the rate of solute transport does not represent a straight line with increasing substrate concentration, but rather, is represented by a hyperbola suggesting saturation of the transporters as substrate concentration increases. As such, mediated transport activities can be examined by Michaelis-Menten parameters analogous to those of saturable, enzyme-catalyzed reactions (Christensen and Kilberg, 1987). Classically, the description of the movement of amino acids by a kinetically distinct transport activity has been termed a transport “system.” To this end, the relative activity of a particular transport system is reflected in the maximal velocity (V_{max}) of transport while the relative affinity of a substrate for a particular transport system can be represented as the concentration of substrate leading to half-maximal velocity (K_m). Transport velocities are typically reported as amount of substrate transported per amount of transporter, approximated by either mg protein or number of cells, per unit time.

Three basic mechanisms exist for the transport of substrates across the plasma membrane. Two transport processes mediate the accumulation of substrate against a concentration gradient, primary active transport and secondary active transport. The third process is driven solely by the "downhill" movement of substrate with its concentration gradient, facilitated transport. It should be noted that facilitated transport is not facilitated diffusion as the former is protein-mediated and demonstrates saturable kinetics. Solute movement by diffusion, on the other hand, increases in a linear fashion with increasing solute concentration and does not show saturation.

Primary active transport is the direct coupling of an energy-generating reaction to the transport of a solute. In most cases, the energy used is the hydrolysis of ATP, and this serves to accumulate solute against its concentration gradient (chemical gradient). If the solute molecule is charged, an electrochemical gradient is generated. This is the case for the transport of Na^+ and K^+ by Na^+/K^+ ATPase. The hydrolysis of ATP drives the transport of $3Na^+$ from cytoplasm to the extracellular medium with the transport of $2K^+$ from the extracellular medium to the cytoplasm. The transport of two substrate molecules across the membrane in opposite directions is referred to as antiport. The Na^+ electrochemical gradient can then be utilized as an energy source for secondary active transport. In this case, the "downhill" flux of the Na^+ can drive the transport of substrate against its electrochemical gradient. The transport of two substrate molecules in the same direction across the membrane is referred to as symport. Facilitated transport represents the movement of solute molecules across the membrane utilizing only the electrochemical gradient of the substrate molecule itself as an energy source for transport. In the case of amino acids, these molecules are transported by either secondary active or facilitated transport. In mammalian cells, no transport of amino acids has been linked to the hydrolysis of ATP, but the Na^+ gradient generated by Na^+/K^+ ATPase is an important driving force for amino acid transport.

Cationic Amino Acid Transport Systems

The identification of a transport system for cationic amino acids distinct from neutral amino acids was defined in Ehrlich ascites tumor cells by Christensen and Liang in 1966. This transport system was originally defined as System Ly^+ to illustrate affinity for lysine. Later the transport system was renamed System y^+ (Bannai et al., 1984), and the system accepts lysine, arginine, ornithine, and histidine when positively charged. Uptake by System y^+ is characterized as Na^+-independent facilitated transport of exclusively cationic amino acids. However, in the presence of Na^+, this system can interact with neutral amino acids as well (Christensen et al., 1969). Although the driving force for this transport is the "downhill" movement of substrate with its concentration gradient, System y^+ is electrogenic and can concentrate amino acids to an extracellular to intracellular distribution profile of greater than one based soley on the membrane potential of the cell (Bussolati et al., 1987). This system is also affected by trans-stimulation (White and Christensen, 1982); high intracellular concentrations of substrate increase the activity of this transporter presumably by decreasing the time required for the reorientation of the transporter such that the substrate binding site faces the extracellular medium. System y^+ mediates the transport of cationic amino acids in virtually every cell type with the exception of the liver (White, 1985). The cDNAs encoding System y^+ activity have been cloned and are described in detail below.

Two additional systems for the transport of cationic amino acids have been described by Van Winkle and coworkers (1985; 1988). The first, System $B^{o,+}$, is a Na^+-dependent secondary active transport system that accepts not only cationic amino acids but neutral amino acids as well (Van Winkle et al., 1988). This system has also been described in *Xenopus* oocytes (Campa and Kilberg, 1991) and in several other cell types including human fibroblasts, porcine aortic endothelial cells, and rat FAO hepatoma cells (Kilberg et al., 1993). System $B^{o,+}$ accepts neutral amino acids with bulky side-chains and branching at both the α and β carbon atoms (Van Winkle et al., 1988). This system is also inhibited by bicyclic amino acids including 2-aminobicyclo[2,2,1] heptane-2-carboxylic acid (BCH), once thought to be specific for the Na^+-independent System L (Christensen et al., 1969). The second system is a Na^+-independent facilitated transporter System $b^{o,+}$. This system also accepts both cationic and neutral amino acids; however, System $b^{o,+}$ does not accept branching of neutral amino acids on the α or β carbon (Van Winkle et al., 1988). This system is also present in *Xenopus* oocytes and a variety of other cell types (Kilberg et al., 1993). An analogous transport system has been identified in erythrocyte membrane vesicles called System y^+L (Devés et al., 1992). The lack of inhibition of System y^+L by the sulfhydryl modification reagent N-ethylmalemide (NEM) appears to discriminate this activity from that of System $b^{o,+}$ (Devés et al., 1993). Additionally, System y^+L mediated the uptake of cationic amino acids in a Na^+-independent manner and neutral amino acids in the presence of Na^+ (Devés et al., 1992). System $b^{o,+}$ mediates the uptake of both cationic and neutral amino acids in a Na^+-independent manner (Van Winkle et al., 1988).

Anionic Amino Acid Transport Systems

A Na^+-dependent, secondary active transport system has been described for the anionic amino acids aspartate and glutamate, System X^-_{AG} (Gazzola et al., 1981). This system transports both the D- and L- steroisomers of aspartate but only L-glutamate and is very sensitive to membrane potential. The mechanism for amino acid uptake through System X^-_{AG} involves the symport of $2Na^+$ with the amino acid and the antiport of a K^+ and either OH^- or HCO_3^- for each transport cycle (Hediger, 1995). Some cell types such as the Ehrlich ascites tumor cell have no specific system for the transport of anionic amino acids but rather mediate the uptake of these substrates in the zwitterionic form through systems for neutral amino acids such as Systems ASC and L (Garcia-Sancho et al., 1977). In the adult rat hepatocyte, however, there appears to be two kinetically distinguishable, Na^+-dependent systems for the uptake of anionic amino acids (Gazzola et al., 1981). Cysteate and cysteinesulfinate effectively inhibit both transport systems in normal hepatocytes. However, upon transformation, cysteate and its homolog homocysteate differentially each system (Gazzola et al., 1981). These data were the first indication that multiple proteins may be mediating similar transport activities, and in fact, several cDNAs each mediating the Na^+-dependent uptake of anionic amino acids have been identified. The cDNAs encoding this activity will be described in detail below.

A Na^+-independent, facilitated transporter also exists for the uptake of anionic amino acids (Guidotti and Gazzola, 1992). System x^-_c mediates the transport of L-glutamate and L-cystine by trans-stimulaton of substrate coupled antiport. In fibroblasts, a glutamine-cystine cycle exists whereby L-glutamine enters the cell via neutral amino acid transport Systems A and ASC (Gazzola et al., 1981). A significant portion is converted intracellularly to glutamate (Bannai and Ishii, 1988). High intracellular levels of glutamate are maintained by deamination of glutamine and by Na^+-dependent glutamate uptake via System X^-_{AG}. Glutamate then effluxes down its concentration gradient through x^-_c driving the uptake of cystine.

Introduction to the Placenta

Normal fetal growth and development requires a continous supply of nutrients from the maternal circulation throughout gestation. The placenta is functionally responsible for the uptake and transport of these nutrients from the maternal to fetal circulations, as well as the removal of waste products from the fetus to be metabolized in the placenta or transported to the mother. As such, Na^+-dependent uptake mechanisms and Na^+-independent efflux mechanisms would be expected on both the maternal and fetal sides of the placenta. Most of these transport mechanisms have been localized to the apical (maternal-facing) and basal (fetal-facing) membranes of the placental syncytiotrophoblast (Sideri et al., 1983; Smith et al., 1992a).

The syncytiotrophoblast is a multi-nucleated, continous epithelial layer resulting from the fusion of terminally differentiated cytotrophoblasts (Sideri et al., 1983). In the placenta of rats and higher primates including humans, the maternal surface of the syncytiotrophoblast is bathed in blood resulting from the breakdown of the maternal circulatory endothelium in the villous tissue. As such, the maternal circulation is in direct apposition to the nutrient transporters on the apical membrane of the syncytiotrophoblast. This type of placenta is termed hemochorial and is distinct from epitheliochorial (pig) and syndesmochorial (sheep) placentas in which uterine tissue lies between the maternal circulation and the trophoblast (Munro, 1986). The only major difference between the rat and human hemochorial placentas is that the human placenta has only a single syncytiotrophoblast layer while the rat placenta has three. The functional significance of these additional layers in the rat has not yet been described.

Placental Amino Acid Transport

Christensen and Streicher first reported in 1948 that the human placenta had the ability to concentrate many amino acids from the maternal to fetal circulation. In addition, the fetal to maternal serum amino acid ratio (F/M) for most amino acids is greater than one, indicating that most amino acids are concentrated in the fetal circulation (Bernardini et al., 1991). The general pattern of F/M is consistent in sheep, guinea pig, human, and rat (Phillips et al., 1978; Marconi et al., 1989). The only notable exception is the anionic amino acids aspartate and glutamate which show significant fetal concentration in the rat but nearly no concentration in all other species (Arola et al., 1982). In general, the trophoblast concentration of amino acids is significantly greater than that of either the maternal or fetal circulations (Lemmons et al., 1976). This suggests that Na^+-dependent concentrative transporters are required on the maternal-facing apical membrane to maintain high intracellular concentrations of amino acids. This also suggests that transfer to the fetus may be mediated by efflux of substrates down the respective concentration gradients.

Schneider and coworkers in 1979 described the establishment of transplacental gradients for various amino acids. Equimolar concentrations of amino acids were added to both sides of the human placenta in a closed perfusion system. After incubation, the F/M ratios of alanine, glycine, and lysine increased significantly indicating concentration by the placenta of these substrates in the fetal circulation. At the same time, the fetal distribution ratios of aspartate and glutamate decreased to near zero demonstrating the clearance of these amino acids from the fetal circulation. This fetal clearance of these substrates was accompanied by an increase in the release from the placenta of asparagine and glutamine leading to the first suggestion of a glutamate/glutamine cycle described below.

Placental neutral amino acid transport. Although the apical membrane of the syncytiotrophoblast has been shown to contain microvilli (Booth and Vanderpuye, 1983; King, 1983), the amino acid transporter complement represents more ubiquitously expressed transporters rather than the transporters associated with the microvilli of the intestine (Stevens et al., 1984). Apical membranes isolated from placentas of nearly all species including rat (Alonso-Torre et al., 1992; Novak et al., in press), demonstrate Na^+-dependent neutral amino acid uptake consistent with System A (Smith et al., 1992). This activity represents the uptake of small neutral amino acids with no branching on the ß-carbon (Christensen et al., 1965). Other Na^+-dependent components of neutral amino acid uptake that may be present in the apical membrane are Systems ASC and N. However, reports noting the absence of these systems in humans have also been described

(Ganapathy et al., 1986; Johnson and Smith, 1988). Systems A, ASC, and the Na^+-independent System L, have all been described on the basal membrane of the human placenta (Hoeltzli and Smith, 1989; Smith et al., 1992a).

Placental cationic amino acid transport. The cationic amino acids arginine and lysine demonstrate high F/M ratios and are avidly concentrated in the placenta and fetus (Bernadini et al., 1991). Perfusion studies have demonstrated transplacental flux of cationic amino acids in guinea pig (Wheeler and Yudilevich, 1989); however, with the exception of the human placenta, the cationic amino acid transporters of either membrane of the trophoblast have not been well characterized, and with regards to the rat placenta, virtually nothing has been reported. Wheeler and Yudelivich (1989), utilizing the perfused guinea pig model, reported "System y^+-like" transport activity within the placenta, but the membrane surface was not identified. Furthermore, this study found some Na^+-dependency to lysine and arginine transport, more characteristic of System $B^{o,+}$ (Van Winkle et al., 1985) than System y^+ (Christensen and Liang, 1966). Also, the cationic amino acid transport observed was never tested for inhibition by leucine to discriminate between System y^+ and System $b^{o,+}$ (Van Winkle et al., 1988) The presence of System y^+ transport activity could not be documented in human placental apical membrane vesicles (Kudo et al., 1987), but was demonstrated by Furesz and coworkers (1991) in basolateral membrane vesicles, along with System $b^{o,+}$

activity. Subsequent studies by the same laboratory identified a Na^+-independent, neutral amino acid-inhibitable cationic transporter on the apical membrane and suggested that it was different from that on the basal membrane (Furesz et al., 1995). My work (Chapter 3) describes the presence of all three cationic amino acid transport systems (y^+, $B^{o,+}$, and $b^{o,+}$) in both apical and basal membrane vesicles from rat placenta.

Placental anionic amino acid transport. In general, the F/M ratios of anionic amino acids are unique from all other amino acids in that the distribution ratio is less than one indicating no concentration in the fetus (Battaglia, 1982). In the rat, however, the distribution ratio is fairly high (Arola et al., 1982). Perfusion studies in the human (Schneider et al., 1979), guinea pig (Stegink et al., 1975), and sheep (Lemmons et al., 1976) have all suggested that glutamate and aspartate are extracted from both the maternal and fetal circulation. While conversion of these anionic amino acids to glutamine and asparagine does occur within the placenta with subsequent efflux to the fetus, the majority of the placental glutamate concentration is oxidixed within the mitochondria (Vaughn et al., 1995). In human membrane vesicles, a Na^+-dependent transport system consistent with System X^-_{AG} has been described on both the apical (Iioka et al., 1985; Hoeltzli et al., 1990) and basal (Moe and Smith, 1989) membranes.

Metabolic Interrelationships Between the Placenta and Fetus

Most amino acids are supplied to the fetus in concentrations that are well in excess of the accumulation of these precursors in fetal protein (Lemons et al., 1976). From this supply, the carbon skeletons of amino acids can be used for oxidation, and this high level of metabolism is reflected in the high level of urea production in the fetus (Gresham et al., 1972). The placenta, also capable of amino acid metabolism but lacking a complete urea cycle, releases a significant amount of NH_3 into the fetal circulation which is then utilized for trans-amination reactions in the fetal liver (Holzman et al., 1977).

Glutamine/glutamate cycle. In a single circulatory passage in the sheep, the basal membrane of the placental trophoblast is able to extract nearly 90% of the glutamate in the fetal circulation (Vaughn et al., 1995). The Na^+-dependent glutamate transport mechanisms on this membrane, as well as on the apical membrane, are responsible for the high placental concentration of glutamate (mM) compared to either the maternal or fetal circulation (μM) (Lemmons et al., 1976). Metabolism of glutamine by fetal tissues is likely the source of this high level of glutamate (Vaughn et al., 1995). Although the majority of glutamate extracted by the placenta is rapidly decarboxylated (Moores et al., 1994), about 10% is returned to the fetus as glutamine by the action of placental glutamine synthetase (Battaglia, 1992). This glutamine is then utilized by the fetus as described above setting up a glutamine/glutamate cycle between the fetus and placenta.

Serine/glycine cycle. Analogous to the situation with glutamate, serine is not concentrated in the fetal circulation by the placenta (Cetin et al., 1991). Rather, serine is extracted from the fetal circulation by the basal membrane of the trophoblast (Cetin et al., 1992). The likely source of this serine is by oxidation of glycine by the fetal liver and extrahepatic tissues. Multiple tracer infusion studies by Moores and coworkers (1990) with [1-^{13}C] and [1-^{14}C] serine have demonstrated that a significant portion of the serine extracted from the fetal circulation in the placenta is converted to glycine and transported back to the fetus. Again, a metabolic cycle appears to be in place for not only the removal of a potentially toxic metabolic byproduct from the fetal circulation but also the reutilization of this precursor in the placenta for delivery of an essential nutrient back to the fetus.

Molecular Biology of Charged Mammalian Amino Acid Transporters

Until 1991, no cDNAs corresponding to kinetically defined α-amino acid transport systems had been described. It was not until the first description of a cationic amino acid transporter cDNA by the laboratory of James Cunningham

(Albritton et al., 1989; Kim et al., 1991) that this field moved into a new era. As the entire course of this study has been accomplished in this new light, a thorough treatment of the molecular advances of charged amino acid transport is included.

The CAT Family for Cationic Substrates

Three members of the CAT family of amino acid transporters have been cloned, each of which mediate the Na^+-independent transport of the cationic amino acids arginine, lysine, and histidine when positively charged. Although there is significant similarity in the deduced amino acid sequence of these proteins and they share a common substrate specificity, the tissue specific expression and apparent role of these proteins in metabolism appears very different.

Cloning of the CAT Family

The cloning of the first member of this family by Cunningham and co-workers resulted from the search for the host cell protein responsible for the infection of certain cell types by the ecotropic murine leukemia virus (Albritton et al., 1989). The infectivity of the virus, and thus the susceptibility of a cell, is mediated by the binding of the virus envelope protein gp70 to a naturally occurring receptor in the plasma membrane of host cells. DNA isolated from susceptible murine cells (NIH 3T3 fibroblasts) was used to stably-transfect a non-susceptible human cell line (EJ bladder carcinoma cells). Recombinant retrovirus conferring

G418 antibiotic resistance upon infection was used to identify EJ cells expressing murine DNA for the receptor. Cloning of the cDNA was then accomplished by Southern blot analysis with mouse repetitive DNA and linkage analysis to the receptor gene.

The deduced amino acid sequence of the cDNA clone, EcoR, revealed a 622 amino acid glycoprotein with a predicted molecular mass of 67 kDa. Interestingly, the hydropathy profile of the protein revealed 12 to 14 membrane spanning regions, similar in structure to the previously cloned facilitated glucose transporters; however, no significant similarity to any other protein in the sequence data bases was demonstrated.

In turn, the human gene homologous to the murine EcoR receptor was cloned and mapped to chromosome 13 (Yoshimoto et al., 1991). The open reading frame consists of 10 introns and 11 exons, and codes for a mRNA transcript of approximately 9 kb representing long untranslated regions. The cDNA sequence predicts a 14 membrane-spanning protein of 629 amino acids with a molecular mass of 68 kDa. The murine and human proteins share 87.6% identity.

A partial rat EcoR cDNA was also isolated (Stoll et al., 1991), and the full-length clone followed (Wu et al., 1994). The cDNA open reading frame codes for a 624 amino acid protein sharing 97% sequence homology with the murine receptor. The most recent clone (Puppi and Henning, 1995) identified 7.0 kb of the 7.9 kb mRNA; however, the significance of the 5 kb 3' untranslated region of the mRNA remains to be revealed.

The identification of a homologous EcoR gene in human cells which are not susceptible to MuLV infection suggested another endogenous function for the receptor. However, the function of the EcoR cDNA as a transporter remained undescribed until two independent groups simultaneously described the cationic amino acid transport properties of the murine cDNA (Kim et al., 1991, Wang et al., 1991).

Upon re-evaluation of the protein sequence data bases, Kim and coworkers (1991) identified sequence similarity between the murine EcoR protein and the arginine and histidine permeases of *Saccharomyces cerevisiae*, suggesting a related function. *Xenopus* oocytes, injected with cRNA corresponding to the murine EcoR, demonstrated significantly higher radiolabelled gp70 binding than water-injected controls consistent with the function of the protein as a receptor for MuLV. The cRNA-injected oocytes also demonstrated saturable accumulation of radiolabelled cationic amino acids L-arginine, L-lysine, L-ornithine, and L-histidine, while the uptake of anionic and neutral amino acids was not different from water-injected controls. The substrate specificity of the cRNA-mediated uptake, as well as the apparent K_m for cationic amino acids, is consistent with the previously kinetically defined transport activity, System y^+ (Christensen and Liang, 1966; White, 1985).

Similar amino acid transport defining properties for murine EcoR were described by Wang and coworkers (1991) in *Xenopus* oocytes utilizing both radiolabelled substrate and electrophysiological measurements of amino acid uptake. While the name EcoR has been applied to the murine protein and Rec-1 applied to the gene when describing its role in viral infection, the cDNA has been termed mCAT1 (murine cationic amino acid transporter 1), when considering its amino acid transport properties

Interestingly, the cloning of the second member of this family of amino acid transporters also came about in the pursuit of genes unrelated to amino acid transporters, as was the case for mCAT1. MacLeod and coworkers (1990a), in an attempt to isolate genes involved in T-cell function and the ability to promote tumor formation in mice, isolated several cDNAs which were expressed at high levels in a highly tumorigenic murine T-lymphoma clone (SL12.4) and at nearly undetectable levels in a non-invasive sister clone. Using probes, enriched in SL12.4 expressed mRNAs by subtractive hybridization, and differential λgt10 cDNA library screening, the same group isolated a cDNA (20.5) which coded for a putative hydrophobic protein showing significant amino acid sequence identity with the previously cloned mCAT1 (EcoR) cDNA (MacLeod et al., 1990b). The 2.4 kb cDNA, termed Tea (T-cell early-activation gene), predicted a 49.6 kDa protein of 453 amino acids with seven membrane-spanning domains. Although clearly related to the mCAT1 gene, the protein was truncated, and the complete product of the Tea gene was not described until the full length cDNA was isolated by the same group (Reizer et al., 1993). The predicted amino acid sequence added an additional 205 amino acids to the N-terminus, contained 12 transmembrane domains, and shared 61% amino acid identity with mCAT1. The Tea gene has been localized to murine chromosome 8 (MacLeod et al., 1990b).

The function of the Tea gene was suggested by structural similarity to a family of bacterial and yeast nutrient transporters (Reizer et al., 1993), and was finally described by Kakuda and coworkers (1993). *Xenopus* oocytes injected with Tea cRNA demonstrated significantly increased uptakes of cationic amino acids compared to water-injected controls. In fact, of all the 20 naturally occurring amino acids, as well as amino acid derivatives used for identification of specific transport systems tested, only the L-cationic amino acids elicited substrate-induced changes in membrane current suggesting transmembrane flux. The transport elicited by the Tea cRNA was saturable and Na^+-independent, similar to the cationic amino acid transport mediated by the mCAT1 cDNA. Although the K_m for arginine determined for Tea and mCAT1 (approximately 200 μM) by Kakuda and coworkers (1993) was two-fold that described for mCAT1 previously (Kim et al., 1991), further analysis of amino acid transport by electrophysiological measurements revealed a similar K_m for both transporters of approximately 150 to 250 μM. A similar characterization of the complete Tea cDNA was also described by Closs and coworkers (1993b). Although some confusion in nomenclature existed due to the rapid isolation of these and homologous transporters by independent laboratories. When considering its amino acid transport properties, the full-length protein product of the Tea cDNA is generally referred to as mCAT2.

Using the partial mCAT2 cDNA provided by the MacLeod laboratory, Closs and coworkers (1993a) cloned a third member of the CAT family from murine liver, mCAT2a. The mCAT2 and mCAT2a cDNAs arise from the same gene on mouse chromosome 8, and the two sequences are identical with the exception of an alternatively spliced region on the predicted fourth extracellular loop. Radiolabelled amino acid uptake in *Xenopus* oocytes injected with mCAT2a cRNA demonstrated significantly higher accumulation of L-cationic substrates, but interestingly, the apparent K_m for arginine was nearly ten-fold higher than that for either mCAT1 or mCAT2 (Closs et al., 1993b) It should be noted that the original designation for this cDNA was mCAT2a and the lymphocyte clone mCAT2b (Closs et al., 1993a). However, it is generally accepted that the liver clone is designated mCAT2a and the lymphocyte clone, mCAT2.

Tissue Specific Expression of the CAT Transporters

In agreement with its function as the previously kinetically defined system y^+, the expression of CAT1 is fairly ubiquitous. The initial cloning of this cDNA from NIH 3T3 cells demonstrated its expression in murine fibroblasts

(Albritton et al., 1989). In murine tissues, the primary transcript size of mCAT1 is 7.9 kb with an additional 7.0 kb transcript, the result of differential polyadenylation (Albritton et al., 1989), seen in some tissues (Kim et al., 1991). Expression is seen in virtually every tissue examined including brain, bone marrow, testis, skeletal muscle, heart, stomach, spleen, kidney, lung, ovary, uterus, intestine, and T-lymphocytes (Kim et al., 1991; Kakuda et al., 1993).

It rats, a primary transcript size of 7.9 kb, with an additional, lower abundance transcript of 3.4 kb seen in some cases, has been demonstrated by our laboratory in rat placenta and hepatoma cells (Kilberg et al., 1993). Transcripts are also seen in rat brain (Stoll et al., 1991), rat intestine (Puppi et al., 1995), and primary culture hepatocytes (Closs et al., 1993b).

Human hCAT1 mRNA expression has been reported as a 9.0 kb and 7.9 kb transcript in human T-cells (Yoshimoto et al., 1991) and human lymphoblastic leukemia, human kidney carcinoma, human lung carcinoma, human colon adenocarcinoma (Yoshimoto et al., 1991), and a 7.9 kb transcript in CaCo2 human intestinal cells (Pan et al., 1995). An immunoreactive protein, detected with an antipeptide antibody to CAT1, was seen on the plasma membrane of human fibroblasts by our laboratory (Woodard et al., 1994) and represents the only published report of the detection of the endogenous CAT1 protein.

The only tissue lacking expression of CAT1 is the liver. Consistent with these data and the lack of mRNA reported by several groups (Kim et al., 1991; Kakuda et al., 1993; Kilberg et al., 1993; Closs et al., 1993; Wu et al., 1994), and by the lack of immunoreactive CAT1 protein by histochemistry in rat and human liver (Woodard et al., 1994).

The expression of the CAT2 gene isoforms is somewhat more limited than the expression of CAT1. The mCAT2 gene was isolated from and is expressed at very high levels in a murine T-lymphoma cell line capable of causing invasive tumors in mice (MacLeod et al., 1990a). Subsequently, mCAT2 expression has been shown to be limited to Concanavalin A activated spleen cells, thymic epithelial cells, and liver cells (MacLeod et al., 1990b). Tissues also demonstrating expression of mCAT2 mRNA include skeletal muscle, skin, lung, brain, uterus and stomach (Kakuda et al., 1993).

Expression of mCAT2a mRNA is even more limited in that detectable levels of this isoform are only detectable in liver (Closs et al., 1993).

Regulation of CAT Transporter Expression

Expression of CAT1 seems to involve the regulation of this transporter when cells, either by naturally occurring developmental changes or inducible means, are shifted from a quiescent, differentiated state to an undifferentiated, rapidly growing state. Using several different model systems, Meruelo and coworkers demonstrated the above (Yoshimoto et al., 1991). First, activation of murine T-cells by Concanavalin A, or B-cells by LPS, increased steady-state mCAT1 mRNA expression by over 10-fold compared to that of quiescent splenocytes or thymocytes. Splenomegaly, induced by mink cell focus-forming virus, also increased the expression of mCAT1 mRNA by over 10-fold. The down-regulation of CAT1 was demonstrated in the HL-60 promyelocytic leukemia cell line as it progresses toward terminal differentiation into granulocytes or macrophages by either DMSO or PMA, respectively. Finally, this group also demonstrated the elevated expression of CAT1 in tumor cells when compared to noncancerous parental cell lines.

Yoshimoto and coworkers (1991) also made initial observations into the mechanisms behind the regulation of CAT1 expression by providing evidence that increased CAT1 expression is modulated by protein kinase C (PKC). Evidence for PKC modulation of CAT1 includes 1) the increase in CAT1 expression when cells are treated with phorbol esters, 2) the failure to increase CAT1 expression by phorbol ester analogs that do not induce the PKC pathway, 3) the inhibition of phorbol ester-induced CAT1 expression by PKC inhibitors, and 4) the induction of CAT1 expression by calcium ionophores.

CAT1 expression is not detectable in normal adult liver (Kim et al., 1991; Kakuda et al., 1993; Kilberg et al., 1993; Closs et al., 1993b; Wu et al., 1994), and consistent with the lack of CAT1 is the inability of MuLV to infect adult hepatocytes (Jaenisch and Hoffman, 1979). It has been demonstrated that infection of liver cells with retroviruses is only possible after partial hepatectomy (Jaenisch and Hoffman, 1979), several groups have focused on the examination of CAT1 expression in models which induce of liver cell division, such as liver regeneration. Closs and coworkers (1993c) found induction of mCAT1 expression in murine hepatocytes after 48 hrs of culture but no detectable CAT1 expression in liver 12 to 48 hrs after partial hepatectomy. The latter result seems to contradict the retroviral infection data. Although Wu and coworkers (1994) also demonstrated no detectable CAT1 mRNA 12 hrs after hepatectomy, there is an induction of CAT1 synthesis at 4 to 6 hrs after hepatectomy, which parallels the time course of viral infection. CAT1 expression in liver can also be induced by other stimuli including insulin, dexamethasone, and excess arginine (Wu et al., 1994). Nutrient regulation of CAT1 in liver has also been demonstrated by induction of CAT1 synthesis in animals chronically fed a low-protein diet (Wu et al., 1994).

Developmental regulation of CAT1 has also been demonstrated. In a published report from the analyses carried out in this study, our laboratory examined the regulation of amino acid transport in rat placenta and found an induction of CAT1 mRNA expression from 14 to 20 days gestation, equilivent to the final trimester of human pregnancy (Malandro et al., 1994). It is thought that this induction in transport is to supply amino acids to accommodate the tremendous increase in fetal growth in late gestation. Our data was novel in that we demonstrated not only an increase in steady-state mRNA levels but also a parallel increase in cationic amino acid uptake, confirming the role of CAT1 as an amino acid transporter as well. Campione and VanWinkle (1994) have also described the developmental regulation of the CAT genes, detecting both mCAT1 and mCAT2 in as early as the one-cell stage of the mouse blastocyst. The appearance of the CAT mRNAs paralleled the appearence of the Na^+-independent, murine blastocyst cationic amino acid transport System y^+. Subsequently, Puppi and Henning (1995) examined the developmental regulation of CAT1 in rat intestine and found high levels of CAT1 in fetal intestine that decreased upon birth and eventually returned to high levels in adulthood.

The most well described analysis of the regulation of the CAT2 gene isoforms led to the cloning of mCAT2. mCAT2 gene expression is undetectable in quiescent splenic T-cells, and enhanced over 15-fold upon activation of those same cells with Concanavalin A (MacLeod et al., 1990a).

Due to the fact that regulation of mCAT2 parallels that of mCAT1 in activated T-cells (Yoshimoto et al., 1991), Kakuda and coworkers (1993) examined the expression of mCAT2 in regenerating liver, a condition regulating the expression of mCAT1 (Wu et al., 1994). No change in mCAT2 steady-state mRNA level was seen in either control, sham-operated, or regenerating liver at 24 hrs after partial hepatectomy (Kakuda et al., 1993). However, it should be noted that increased expression of mCAT1 could not be seen at 24 hrs after hepatectomy as well (Closs et al., 1993), but enhanced expression was seen from 4 to 6 hrs after hepatectomy (Wu et al., 1994), a time point not examined for mCAT2. It still remains to be seen if the transient increase in mCAT1 expression is accompanied by a transient increase in mCAT2 at these earlier time points. Interestingly, regulation of mCAT2 was seen in skeletal muscle following either hepatectomy, splenectomy, or fasting (Carol MacLeod, personal communication). It is postulated that this increase in transporter is to allow for the efflux of amino acids from the muscle, a major source of amino acids during surgical trauma and fasting (Souba, 1987; Rennie et al., 1990).

Genomic Regulation of CAT2 Transporter mRNA Splicing

The regulation of expression of the mCAT2 gene isoforms involves various mRNA splicing events in both the coding and non-coding regions of the gene. As was previously mentioned, two different mRNA isoforms are produced from the mCAT2 gene in a tissue specific manner. The mCAT2 and mCAT2a predicted protein products differ in only a single stretch of 41 or 40 amino acids, respectively, in the 4th extracellular loop of the transporter (Kakuda et al., 1993; Closs et al., 1993a).

Analysis of many cDNA clones of mCAT2 from T-lymphoma cells by MacLeod and coworkers, led to the identification of 4 splicing isoforms which contain the entire coding sequence but differ in 5' untranslated regions (Finley et al., in press). An independent laboratory isolated a fifth splicing isoform (Kavanaugh et al., 1994). Each of these mRNA converge into a common sequence 16 base pairs upstream of the initiation AUG start codon. The mCAT2 gene 5' untranslated exons span a distance of over 18 kb which is thought to contain up to 5 distinct promoters (Finley et al., in press). The sequence upstream of the first untranslated exon contains no TATA sequence but DNA binding motifs common to TATA-less promoters including GC-rich sequences and SP1 binding sites, and it is the product of this promoter that is found in all cell types that express mCAT2. The sequence upstream of the second untranslated exon is a classic TATA promoter. The products of this and the other putative promoters are likely responsible for the complex tissue specific expression of the mCAT2 gene.

Structure and Functional Analysis of the CAT Transporters

Wang and coworkers (1991) have demonstrated in voltage clamped *Xenopus* oocytes injected with mCAT1 cRNA, an inwardly-directed, saturable current in the presence of substrate. When the magnitude of the current is compared to transport of radiolabelled substrate in similarly injected oocytes, movement of one positive charge per molecule of substrate into the cell is seen. Kavanaugh (1993) examined the change in membrane current resulting from CAT1-induced transport, and at membrane potentials ranging from +20 mV to -120 mV, arginine uptake followed values predicted by Michaelis-Menten kinetics. Interestingly, at concentrations of arginine from 0.01 to 1 mM, influx increased exponentially with membrane hyperpolarization rather than demonstrating saturation kinetics. This result provided evidence that the rate limiting step in transport, thought to be the reorientation of the transporter to the outward facing position, is voltage dependent. If the conformational change resulting in the transition of the unliganded receptor to the external membrane conformation were not associated with any charge movement, then steady-state flux would approach a maximum value determined by the rate-limiting voltage-independent step for this transition, and saturation of the transporter at hyperpolarized potentials would be observed. These results suggest that a charge translocation occurs during the reorientation of the unliganded receptor, and that this charge movement significantly affects the voltage-dependence of steady-state cationic amino acid flux mediated by mCAT1. These data also provide a fundamental difference in the function of mCAT1 with respect to transport mediated by the Na^+-dependent glucose and γ-aminobutyric acid transporters, both of which approach maximal rate at hyperpolarized potentials (Kavanaugh et al., 1994).

In an effort to determine the structural features responsible for the differences in transport activity between mCAT1 and mCAT2/mCAT2a, Closs and coworkers (1993b) examined the expression of chimeric proteins in *Xenopus* oocytes. mCAT1, previously described in a similar system, has a high substrate affinity and is capable of trans-stimulation by increased concentrations of substrate on the opposite side of the membrane (Closs et al., 1993a), similar transport properities have been demonstrated for mCAT2 (Closs et al., 1993b; Kakuda et al., 1993). However, mCAT2a, the liver-specific mCAT2 isoform, has a 10-fold greater apparent K_m for arginine than mCAT1 or mCAT2 but a higher V_{max} (Closs et al., 1993a). The only difference between mCAT2 and mCAT2a is a strech of 40 or 41 amino acids contained within a putative extracellular domain. The substitution of this region from mCAT2a into mCAT1 resulted in a greater K_m and V_{max}, consistent with the properties of mCAT2a. The reciprocal construct of mCAT1 into mCAT2a resulted in a K_m and V_{max} consistent with mCAT1.

An examination of critical amino acid residues for the function of mCAT1 was provided by Kabat and coworkers (Wang et al., 1994). The authors observed the conservation of a glutamate residue (position 107 in mCAT1) residing in a putative membrane-spanning region of the transporter. This residue was not only conserved when comparing mCAT1 to mCAT2 and mCAT2a, but also in the arginine and histidine permeases from yeast. The conservative substitution of aspartate (Asp) for glutamate (Glu) at position 107 was not thought to alter the overall structure of the transporter. This fact was supported by the observation that the transporter reached the plasma membrane and bound gp70. However, the Asp-Glu substitution led to the complete abolishment of amino acid transport activity.

Recent reports on the function of mCAT1 and mCAT2 have led to some interesting findings that may force investigators to reevaluate the "one protein-one activity" paradigm. Van Winkle and coworkers (1995) used *Xenopus* oocyte expression, as well as the analysis of previously published data by other investigators, to kinetically examine the transport acitivity mediated by mCAT1 and mCAT2. Interestingly, when electrophysiological measurements were used to assay transport, the L-arginine uptake mediated by both mCAT1 and mCAT2 demonstrated biphasic Eadie-Hoffstee plots indicating at least two kinetically distinct transport activities. Both activities could either be mediated by a single CAT protein, or the CAT protein could be active in addition to upregulating the activity of an endogenous oocyte transporter. Indeed, a major transport system functioning in the oocytes for the transport both cationic and neutral amino acids is upregulated by certain cRNAs (described below).

The Glutamate Transporter Family

Even before the cloning of the glutamate transporter family members, it was suggested that there would be several members to this family as glutamate transport in synaptosomal preparations from different regions of the brain were found to be differentially sensitive to pharmacological agents such as dihydrokainate and L-α-aminoadipate (Hediger, 1995). However, unlike the CAT family where the cloning of the initial two members of the family was by chance, the cloning of the members of the glutamate family came from a concerted effort involving protein purification, antibody production, library screening, and expression cloning for the identification of the involved cDNAs.

Cloning of the Glutamate Family

The first member of this family, GLAST, was isolated by Stoffel and coworkers (Storck et al., 1992). During the isolation of a galactosyltransferase from the rat brain, a hydrophobic protein of about 66 kDa was copurified, which, upon deglycosylation with endoglycosidase F, yielded a protein of 60 kDa. Proteolytic fragments of this protein were then sequenced, and oligonucleotide probes were generated for sequencing of a rat brain cDNA library. A 3 kb clone was isolated which coded for a protein of 543 amino acids with a predicted molecular mass of 59.7 kDa. This cDNA showed considerable sequence homology to the previously cloned glutamate and carboxylate transporters of bacteria. The predicted protein sequence also showed similar structural features to other transport proteins including multiple membrane spanning domains and multiple glycosylation sites. However, the GLAST sequence shows only six well defined putative transmembrane domains at the N-terminus. The C-termnial portion contains six short hydrophobic stretches which are conserved in the prokaryotic transporters as well. This unique membrane topology is diffrent from the CAT transporters described above which have at least 12 well defined predicted membrane-spanning regions, and the description of this topology remains the subject of investigation. To determine the function of the GLAST cDNA, *Xenopus* oocytes were injected with cRNA and transport assayed by uptake of radiolabelled substrate (Storck et al., 1992). Transport of both glutamate and aspartate was demonstrated to be Na^+-dependent and inhibited by DL-threo-3-hydroxyaspartate, the strongest known inbibitor of Na^+-glutamate transport in brain slices (Eisenberg et al., 1984). The human homolog of the GLAST transporter was subsequently isolated, by two independent laboratories, from human brain by low stringency screening of cDNA libraries by Kawakami and coworkers (hGluT, Kawakami et al., 1994) and by Arriza and coworkers (EAAT1, Arriza et al., 1994). The human GLAST shares 97% amino acid sequence identity with the rat transporter. It should be noted that in this manuscript the original clone name of GLAST1 will be used in reference to this transporter isoform from either rat or human not only to credit the original isolation of this clone but also to eliminate ambiguity which may exist particularly with respect to GluT, a designation that has been universally established for facilitated glucose transporter clones.

The second (GLT1) and third members (EAAC1) of this family were isolated shortly after GLAST by Pines and coworkers (1992) and Kanai and Hediger (1992), respectively. The cloning of GLT1 esentially began several years before the isolation of the cDNA with the purification to near-homogenity of the GLT1 tranport protein (Danbolt et al., 1990), and the subsequent generation of both polyclonal (Danbolt et al., 1992) and monoclonal antibodies (Hees et al., 1992). The polyclonal antibody was used to immunoscreen a cDNA expression library, and a 4.6 kb positive clone (GLT1) was isolated (Pines et al., 1992). The cDNA predicted an open reading frame of 1719 bp coding for 573 amino acids with a molecular mass of 63 kDa. The hydropathy profile of GLT1 is similar to that of GLAST1 in that it predicts 6 to 8 putative membrane spanning regions at the N-terminus and several smaller hydrophobic regions at the C-terminus. GLT1 shares homology with prokaryotic glutamate transporters and shares 44.4% amino acid sequence identity and 67.6% homology with GLAST1. Transport assays, to confirm the function of the GLT1 cDNA, were carried out by the expression of the cDNA in mammalian cells and the subsequent transport of radiolabelled substrate. Transport of L-glutamate was Na^+-dependent and stereospecific in that the uptake was completely inhibited by L-glutamate and not D-glutamate. Transport of other neurotransmitters was not mediated by this cDNA as evidenced by the lack of inhibition with GABA, dopamine, noradrenaline, and serotonin. The activity mediated by this cDNA was dependent on internal K^+ but was not inhibited by the K^+ ionophore valinomycin, suggesting the role of K^+ as a substrate rather than the K^+ gradient being the driving force for transport of glutamate. Arriza and coworkers (EAAT2, Arriza et al., 1994) and Manfras and coworkers (GLTR, Manfras et al., 1994) have each identified the human homolog of GLT1. Although some small sequence discrepancies exist, these cDNAs most likely arise from the same gene and share 90.2% amino acid sequence identity with the rat GLT1. Another human clone was isolated by Shashidharan and coworkers (GLAST2, Shashidharan et al., 1994). However, this clone had sequence variations representing a deletion of the first 10 amino acids compared to the rat sequence and two single base deletions which introduces a frame-shift mutation for amino acids 253 - 281 in the human sequence. The remainder of the sequence is identical to the two other human clones. These investigators isolated 5 additional clones from two independently prepared cDNA libraries and obtained sequences identical to the reported cDNA confirming their original observations. As was discussed above, mCAT2 and mCAT2a arise from the same gene and differ in only 41 amino acids resulting from a differential splicing event. Hence, additional experimentation is required to determine if an analogous situation exists with the GLT1 transporter. A mouse homolog of GLT1 was also isolated by Kirschner and coworkers (EAAT2, Kirschner et al., 1994) and has been localized to mouse chromosome 2. It is interesting to note that this transporter maps near two quantative trait loci that are related to mouse models of epilepsy and hyperexcitability. This result could suggest that the abnormal transport of this neurotransmitter may be involved in these phenotypes. Again, as with the GLAST1 transporter, the GLT1 transporter isoform will be referred to in this manuscript as GLT1 either from rat, human, or mouse to eliminate ambiguity.

The third member of the glutamate transport family was identified from rabbit intestine by Kanai and Hediger (1992). A *Xenopus* oocyte expression cloning system was used to express mRNA isolated from rabbit small intestine, and a peak mRNA size fraction was reverse transcribed and used to generate a plasmid cDNA library. A single cRNA was subsequently identified, injected into oocytes, and radiolabelled substrate transport measured. A cDNA (EAAC1) of 3.4 kb was isolated with an open reading frame coding for a protein of 524 amino acids with a predicted molecular mass of 57 kDa. The EAAC1 isoform shares 65.6% amino acid sequence similarity with GLAST1 and 60.8% with GLT1. Expression of EAAC1 resulted in the increase in saturable Na^+-dependent transport of radiolabelled L-glutamate over 1000-fold greater than that of water injected oocytes. Electrophysiological analysis of transport revealed high-affinity K_m's for L-glutamate and L-aspartate in the low micromolar range (12 to 6 μM) and also demonstrated the inhibition of this uptake by D-aspartate and DL-threo-B-hydroxyaspartate, two strong inhibitors of synaptosomal glutamate uptake (Rauen et al., 1992). Subsequently, both Arriza and coworkers (EAAT3, Arriza et al., 1994) and Kanai and coworkers (EAAC1, Kanai et al., 1994) have identified an identical clone as the human homolog of EAAC1 which shares 95.6% amino acid sequence similarity with the rabbit EAAC1.

Hediger and coworkers have localized the human EAAC1 to chromosome 9p24 (Smith et al., 1994). Our laboratory in collaboration with Bob Fremeau (Duke University) has isolated the rat homolog of EAAC1 which shares 94.6% similarity with the rabbit clone (Chapter 4).

Recently, a fourth member of this family has been isolated by Fairman and coworkers (1995) using degenerate primers from the other members of the glutamate family in low stringency PCR from human cerebellum mRNA. The clone (EAAT4) shares amino acid sequence identity with the other human clones identified by the Amara laboratory on the order of 65% (human GLAST), 41% (human GLT1), and 48% (human EAAC1). Expression of EAAT4 in oocytes demonstrated significantly increased, saturable L-glutamate uptake when using either radiolabelled amino acid or electrophysiological measurements to assay transport. Interestingly, substitution of extracellular gluconate for chloride completely inhibited the outwardly-directed current induced by L-aspartate at depolarizing membrane potentials suggesting a role for chloride in transport. However, when membrane potentials were clamped at -60 mv, the substrate-induced current was not significantly different either in the presence or absence of chloride. The data are consistent with a functional model for EAAT4 as a chloride channel activated by substrate (glutamate/aspartate and sodium) binding. This unique property of EAAT4 may be involved in not only the re-uptake of glutamate from the synapse, as is the case for the other glutamate transporters, but also may aid in the re-establishment of membrane potential by cellular chloride permeability.

Distribution of the Glutamate Transporters

In the initial identification of rat GLAST1, mRNA analysis by Northern blotting detected a single mRNA species of 4.5 kb with expression exclusively in the brain (Storck et al., 1992). In situ hybridization of frozen brain sections was performed with the same probe and revealed a low-level, uniform distribution across the entire cerebellum with high-level expression seen only in the cerebellar cortex (Storck et al., 1992). Upon futher analysis, GLAST1 mRNA in the cerebellum was seen in only the Perkinje cell layer. In situ hybridization of rat retina by Otori and coworkers (1994) revealed expression in retinal glial cells, specifically Müeller cells and astrocytes. Rat GLAST protein has been localized by immunoblotting, with a polyclonal antisera generated from the cloned rat sequence, as a broad band with a molecular mass of approximately 66 kDa (Lehre et al., 1995). Expression of the protein correlates well with the expression of the mRNA such that GLAST1 protein is seen primarily in the molecular layer of the cerebellum with expression being restricted to astrocytes. No GLAST1 protein is detected in neurons. Interestingly, human GLAST1 appears more ubiquitously expressed than rat GLAST1. A single mRNA species of approximately 4.0 to 4.2 kb is detected not only in brain but also in heart, lung, placenta, and skeletal muscle (Kawakami et al., 1994; Arriza et al., 1994). In the brain, the expression of human GLAST1 mRNA is abundant in motor cortex, frontal cortex, hippocampus, basal ganglia, and cerebellum (Arriza et al., 1994).

Similar to that of GLAST1, the initial descriptions of rat GLT1 transporter mRNA expression revealed a 10 kb single mRNA species whose expression was restricted to the brain (Pines et al., 1992). The human homologs of GLT1, with a similar mRNA size, were also predominantly brain-specific, with a very weak but detectable signal in placenta (Shashidharan et al., 1994, Arriza et al., 1994). Brain-specific GLT1 mRNA expression was seen at high levels in the motor cortex, frontal cortex, hippocampus, and basal ganglia with lower levels in the cerebellum (Arriza et al., 1994). The mouse GLT1 mRNA is expressed similarly to that of both the rat and human homologs. However, a weak but detectable band is also observed in the liver (Kirschner et al., 1994). The rat GLT1 protein has been localized by immunocytochemical methods using a polyclonal antisera to glial cells, specifically the astrocytic processes (Danbolt et al., 1992), and is expressed at highest levels in the hippocampus, lateral septum, cerebral cortex, and striatum (Lehre et al., 1995). No GLT1 protein was seen in glutaminergic nerve terminals (Danbolt et al., 1994; Lehre et al., 1995). Although the GLAST and GLT1 transporter isoforms have a similar distribution in the brain, the distribution of these two transporters in the retina, a major site of glutaminergic nerve innervation, may significantly differ. GLAST mRNA expression has been localized to Müeller cells and astrocytes (Otori et al., 1994), while GLT1 expression is absent from these cell types as detected by immunocytochemistry (Rauen and Kanner, 1994).

By far, EAAC1 is the most widely expressed of the glutamate transporter isoforms. The rabbit EAAC1 is expressed as 3.5 kb and 2.5 kb mRNA species in small intestine, kidney, brain, liver and heart (Kanai and Hediger, 1992), with a similiar distribution in the human (Arriza et al., 1994; Kanai et al., 1995) and in the rat (Velaz-Faircloth et al., in press). The brain-specific expression of human EAAC1 reveals nearly equal expression in the motor cortex, frontal cortex, hippocampus, basal ganglia, and cerebellum (Arriza et al., 1994). In situ hybridization performed by our laboratory reveals expression of rat EAAC1 in the cerebellar granule cell layer, hippocampus, superior colliculus, and neocortex (Velaz-Faircloth et al., in press). In all cases, EAAC1 mRNA expression was localized to neuronal cell bodies. These data are consistent with the suggestion that GLAST1 and GLT1 represent glial-specific isoforms while EAAC1 represents a neuronal-specific isoform (Kanai and Hediger, 1992).

The EAAT4 isoform mRNA is expressed as a single mRNA species of 2.4 kb and is expressed exclusively in the brain and placenta (Fairman et al., 1995). Northern blot analysis demonstrates exclusively cerebellar expression. However, rtPCR analysis can detect low levels of EAAT4 in brain stem, cortex, and hippocampus.

Regulation of Glutamate Transporter Expression

Developmental regulation glutamate transport in the brain was provided before any of the transporters were specifically identified. Utilizing *Xenopus* oocyte expression of mRNA isolated from developing rat brain, Blakely and coworkers (1991) using pharmacological inhibitors of transport examined the regulation of transport from fetus to adult. Glutamate transport demonstrated a region-specific increase in transport activity throughout development. The suggestion of different proteins mediating glutamate transport in different regions of the brain was also provided by the differential sensitivity of transport mediated by mRNA from different regions of the brain. Specific mRNA sizes were also analyzed which correspond to the subsequently cloned transporter isoforms.

Otori and coworkers (1994) examined the regulation of glutamate transport during ischemia in the rat retina. As was previously mentioned, GLAST1 mRNA expression is primarily limited in the retina to cells in the inner nuclear layer expressing glial fibrillary acidic protein (GFAP), primarily Müeller cells and astrocytes. Retinal ischemia, mediated by the ligation of the central renal artery for 90 minutes then the subsequent reperfusion of the retina for 48 hours, causes neuronal cell death in the inner nuclear layer. However, the glial cells expressing GLAST1 mRNA remain and express the transporter at higher levels. Glutamate transport, possibly mediated by GLAST1 may operate in a reverse mode under ischemic conditions, thus allowing glutamate efflux rather than uptake (Atwell et al., 1993). The authors suggested that the increased GLAST1 would then be available during reperfusion to clear the high levels of extracellular glutamate remaining from the period of ischemia.

The regulation of glutamate transport via GLT1 was described by Casado and coworkers (1993). In vitro, GLT1 purified from porcine brain served as a substrate for protein kinase C leading the investigators to examine glutamate transport activity in a rat glioma cell line (C6) and a HeLa cell line transiently expressing the GLT1 transporter. Upon treatment of both cell lines with phorbol ester, there is a rapid increase (30 min) in L-glutamate transport which follows a similar time course as the phosphorylation of the GLT1 protein. Further evidence for the role of phosphorylation was provided by the mutation of the single protein kinase C consensus phosphorylation site. Site-directed mutation of serine 113 to asparagine did not affect the basal transport rate or transporter number assayed by immunoblot analysis. However, the phorbol ester-mediated increase in glutamate transport was abolished.

Plakidou-Dymock and coworkers (1993) described the regulation of glutamate transport by amino acid deprivation in the bovine renal epithelial cell line NBL-1. The regluation of neutral amino acid transport via System A is well described in many tissues and cell types (Kilberg et al., 1993). The authors used a similar method to study the regulation of glutamate transport by the removal of all extracellular amino acids. Glutamate transport increased in V_{max} by a single kinetically defined system analogus to the previously defined System X^{-}_{AG} (Gazzola et al., 1981). Probes generated to the rabbit EAAC1 sequence by low-stringency PCR identified a single mRNA species consistent with this isoform. However, the regulation of steady-state mRNA level does not correspond to the regulation of glutamate transport in these cells. In fact, the steady-state mRNA level decreases during the period of increased glutamate transport. In this case, one would suggest either a regulation of the transporter at the post-transcriptional level of the presence of another of the transporter isoforms mediating the observed glutamate transport activity.

Structure/Function Relationships of the Glutamate Transporters

All members of the glutamate transport family share a similar structure of at least six transmembrane spanning domains at the N-terminal portion of the protein and at least two conserved putative glycosylation sites located on the extracellular loop between transmembrane segments three and four. Conradt and coworkers (1995) examined the potential role of glycosylation of the GLAST1 transporter by elimination of the two glycosylation sites by site-directed mutagenesis. Comparison of the wild-type and mutant transporters revealed that both of the putative glycosylation sites were involved in N-linked glycosylation. However, replacement of the asparagine residues at these sites with threonine resulted in completely functional and properly targeted transporter.

In an effort to elucidate the residues in the GLT1 protein essential for glutamate transport activity, Zhang and coworkers (1994) utilized site-directed mutagenesis to change two amino acid residues completely conserved in this family. Lysine 298 and histidine 326, both residues in predicted membrane-spanning regions, were substituted with small hydrophilic and charged amino acids. Substitutions in GLT1 of arginine or histidine for lysine result in functional proteins that retain nearly full activity. However, less conservative substitutions of glutamine and threonine at the same position result in a significant decrease in transport activity. These changes were demonstrated to arise from targeting defects of these two mutants as reconstitution of these proteins, prepared from whole cell extracts rather than plasma membranes, into proteoliposomes showed essentially the same activity as the wild type in this assay. However, short of immunofluorescence of the plasma membranes to determine if the mutant transporters reach the plasma membrane, the interpretation of the reconstitution data is limited. In contrast to the lysine mutants, any substitution for the histidine residue at position 326 resulted in a completely non-functional protein. These data suggested that histidine

326 is essential for the function of GLT1 and possibly was involved in substrate binding or translocation. In an additional analysis of the GLT1 transporter, Pines and coworkers (1995) used site-directed mutagenesis to examine the function of conserved negatively-charged amino acid residues located in the hydrophobic portions of the C-terminus of the transporter. Mutation of three of the five conserved residues (aspartate 398, aspartate 470, glutamate 404) resulted in a significant reduction in activity even when these residues were substituted with conserved amino acids of the same charge. Interestingly, substitution of glutamate 404 not only reduced the glutamate transport activity of this transporter, but changed the substrate specificity as well in that both D- and L-aspartate were accepted as substrates (80% of wild-type activity). As the binding of glutamate was unaffected in this mutant, these data suggest a possible selective impairment of glutamate translocation or release on the opposite side of the plasma membrane.

Vandenberg and coworkers (1995), using electrophysiological measurements of transport activity in *Xenopus* oocytes, identified an inherent ion flux, in the absence of glutamate, associated with the expression of human GLAST (EAAT1) but not GLT (EAAT2). A chimeric transporter was made by the substitution of residues 366 - 441 from GLT onto a GLAST backbone. The substituted segment differs in only 18 amino acid residues from the corresponding segment of GLAST.

The chimeric transporter bound kainate with high affinity, a property of the substituted domain of GLT exclusively. The kainate binding also inhibited the constitutive ion flux of the chimeric transporter. This activity was attributed to an uncoupled flux of cations across the plasma membrane. Differential activity of the GLAST versus GLT transporters is also seen in the response of each transporter to arachidonic acid, a messenger molecule involved in ischemia and released upon activation of glutamate receptors (Zerangue et al., 1995). Arachidonic acid inhibits glutamate transport via GLAST by decreasing the transport velocity by about 30%. In contrast, arachidonic acid stimulates transport via GLT by increasing the affinity of the transporter for substrate by about two-fold. Taken together, these data suggest that although the glutamate transporter family is highly homologous, up to 80% in some regions, differential regulation and modulation of transport activity is essential for normal brain function.

Associated Members of the Glutamate Transporter Family

The Kilberg laboratory, in collaboration with Fremeau and coworkers at Duke University (SATT), and Amara and coworkers (ASCT1) independently isolated a cDNA from human brain that shares near 40% sequence identity with the members of the glutamate transport family (Shafqat et al., 1993; Arriza et al., 1993). Although some sequence discrepancy existed, publication of a revised sequence indicated that the two clones were in fact identical (Kilberg et al., 1994).

The cDNA identified an open reading frame of 532 amino acids with a molecular size of 56 kDa. Analogous to GLAST, GLT, and EAAC1, the ASCT1 sequence predicts six well defined transmembrane sequences near the N-terminal of the protein and up to six additional hydrophobic stretches near the C-terminal. However, Arriza and coworkers (1993), again utilizing Xenopous oocyte expression and subsequent electrophysiological assays of transport activity, demonstrated Na^+-dependent uptake not of negatively charged amino acids but of neutral amino acids including alanine, serine, and cysteine. The data presented utilizing transient expression of the transporter in mammalian cells by recombinant vaccinia virus and subsequent assay of transport activity by radiolabelled substrate, gave similar results with the exception of cysteine (Shafqat et al., 1993), a discrepancy that was later resolved (Kilberg et al., 1994). These data were consistent with the previously kinetically defined System ASC (Christensen et al., 1967). System ASC activity has been identified as a high activity transporter in nearly every cell type tested. The mRNAs (approximately 5.0, 3.5, 2.2 kb) for ASCT1 is expressed in high levels in only the brain, skeletal muscle and pancreas. This may suggest an analogous situation to the CAT transporter family described above, in that a similar activity may be mediated by different gene products in different tissues.

A unique property of the kinetically defined system ASC is the ability to accept negatively charged substrates at low pH (Makowske and Christensen, 1982; Vadgama and Christensen, 1984). When System ASC activity is assayed at neutral pH, uptake of primarily neutral amino acids are observed. However, decreasing the extracellular pH results in competitive inhibition by anionic amino acids as well. Use of anionic amino acid analogs cysteate or cysteinesulfinate, with pK values near 1, eliminates titration of the substrate to neutral charge. Tamarappoo and coworkers (in press), utilizing expression of ASCT1 in HeLa cells, observed that ASCT1-mediated activity was inhibited at low pH by anionic amino acid analogs further confirming the assignment of System ASC activity to the ASCT1 clone.

The rBAT and 4F2hc Family of Transporters

One of the most unique families of transport proteins are those of the rBAT and 4F2hc family. These proteins up-regulate the transport of some neutral and positively charged amino acids when their cRNAs are injected into *Xenopus* oocytes. However, the predicted structure of this family is very different from the other transport families in that these proteins are predicted to have only one or four membrane spanning regions instead of the 10 to 12 predicted in the other transporter families. This has led some investigators to suggest these proteins function as regulators or subunits of transport activity, and the expression of these proteins in oocytes serves to induce an already present oocyte transporter. Given either the role of transport or regulatory protein, it is clear that a defect in this protein has profound effects in the kidney leading to the disease cystinuria.

Cloning of the rBAT/4F2hc Family

As was mentioned above, the members of this family were identified by expression cloning strategies in *Xenopus* oocytes. The initial observations from several laboratories of the induction of cationic and neutral amino acid transport in oocytes expressing size-fractionated kidney mRNA, laid the foundation on which the members of this family were cloned (Tate et al., 1989; Bertran et al., 1992b; Magagnin et al., 1992). Expression cloning from three independent laboratories led to the identification of a new class of proteins identified in the kidney from rat (NAA-Tr, Tate et al., 1992; D2, Wells and Hediger, 1992), rabbit (rBAT, Bertran et al., 1992) and human (rBAT, Bertran et al., 1993; D2, Lee et al., 1993). It should be noted that the human sequence reported by Lee and coworkers (1993) appears to lack 22 amino acids of the C-terminus present in the other proteins from rat and rabbit and a similar human clone. Each of the proteins share 80 to 85% sequence identity and a similar structure. We will use the nomenclature of Palacin (1994) by referring to all of these clones as rBAT (related to Basic Amino acid Transport).

The rBAT cDNA predicts an open reading frame of 2049 nucleotides coding for a protein of 683 amino acids with a molecular mass of 77 to 79 kDa. The rBAT protein contains seven putative glycosylaton sites, and a leucine-zipper motif is seen in the rat protein from amino acids 548 to 569 (Wells and Hediger, 1993). Most notably, however, is the unique structure of this protein. Hydrophobicity profiles of rBAT revealed either four (Tate et al., 1992) or one (Wells and Hediger, 1992; Bertran et al., 1992; Bertran et al., 1993; Lee et al., 1993) transmembrane domains. Given the high degree of identity between these proteins, the apparent discrepancy most likely arises from the hydropathy computer program used to predict transmembrane segments rather than from a fundamental difference in protein structure. Several investigators are using molecular approaches to address the topology of this family of proteins and will be discussed below. This structure is in striking contrast to all other nutrient transporters described in which 10 to 12 transmembrane domains are expected. Even in the glutamate transporter family in which only the first six transmembrane segments are well placed, there are at least four to six other hydrophobic streches in the proteins that may possibly be membrane bound. The only class of proteins that share structural features with rBAT is the ligand-gated ion channels including the nicotinic acetylcholine and GABA receptors. These proteins, however, have an N-terminal signal sequence which is cleaved from the mature protein such that the N- and C- termini of these proteins are located extracellularly. As such, the rBAT protein appears to define a new class of membrane proteins.

The gene for the human rBAT was localized to chromosome 2-p21 (Lee et al., 1993; Yan et al., 1994). The functional promoter of the rat rBAT gene has also been identified (Yan et al., 1994).

Expression of the rBAT cRNA in oocytes results in significantly increased the Na^+-independent uptake of both neutral (including alanine, phenylalanine, leucine, and cysteine) and cationic amino acids (lysine and arginine) over water-injected controls. From these data, the Tate and coworkers (1992) concluded that rBAT mediated transport similar to the previously kinetically defined system L (Oxender and Christensen, 1966). However, several features of the cRNA-mediated amino acid uptake are not consistent with this system. First, BCH, a model substrate for system L, does not inhibit neutral amino acid uptake in the injected oocytes. Second, there is no increase in the transport of tryptophan, a high affinity substrate for system L, and lastly, there is significant inhibition of neutral amino acid uptake by cationic amino acids. These data are more consistent with uptake similar to the kinetically defined system $b^{o,+}$, a transporter of both neutral and cationic amino acids (Van Winkle et al., 1988). This system is expressed at high levels in the oocyte and serves as the major upake mechanism for endogenous cationic amino acid uptake in oocytes (Campa and Kilberg, 1991). This fact, along with the unique structure of rBAT, has led some investigators to suggest that the rBAT protein may be interacting with an endogenous oocyte transporter, inducing system $b^{o,+}$ uptake rather than functioning as a transport protein itself. Short of purification of the rBAT protein to homogeneity and reconstitution into proteoliposomes, this question will remain unanswered.

Two laboratories identified sequence similarities between the rBAT proteins and a previously cloned protein 4F2hc. The 4F2hc protein was identified in a human T-cell line as a heterodimer composed of an 85 kDa glycosylated heavy chain, which spans the membrane a single time, in disulfide linkage with a 45 kDa nonglycosylated light chain (Eisenbarth et al., 1980; Hemler et al., 1982). The cDNA was subsequently isolated from both mouse and human (Quackenbush et al., 1987; Teixeira et al., 1987; Parmaceck et al., 1989), and the 4F2 heavy chain (4F2hc) was found to share 15% amino acid sequence identity with the rBAT protein. The similarity with the rBAT proteins led Bertran and coworkers (1992b) and Wells and coworkers (1992) to investigate amino acid transport activity associated with 4F2hc. Injection of 4F2hc cRNA into oocytes resulted in the induction of Na^+-independent cationic amino acid uptake over water-injected controls. Neutral amino acid transport was also induced but in a Na^+-dependent rather than Na^+-independent manner. Also of note is the fact that the expression of 4F2hc cRNA did not induce the transport of cysteine, suggesting a fundamental difference between the activities of rBAT and 4F2hc. The activities induced by 4F2hc more closely resemble the previously kinetically defined y^+L identified in human erythrocytes (Devés et al., 1992). System y^+L transport is characterized as high affinity, Na^+-independent cationic and Na^+-dependent neutral amino acid uptake. However, 4F2hc could also be interacting with System y^+, which mediates Na^+-independent cationic amino acid transport and has been shown to interact with neutral amino acids in the presence of Na^+ (Christensen et al., 1969b). Interestingly, the expression of 4F2hc in T-cells and the possible interaction with cationic amino acid transporters could suggest further examination of the inducible expression of mCAT2 and its possible interaction with 4F2hc.

Tissue Distribution of rBAT

rBAT mRNA is expressed in various tissues as a 2.2 to 2.5 kb species and a 3.5 to 4.4 kb mRNA species. Both of the mRNA species identified and expressed in oocytes from rabbit kidney mRNA (Markovich et al., 1993). Both transcripts can induce transport in a similar fashion, and the difference in size was demonstrated to be differential polyadenylation of the transcripts. rBAT mRNA, as assayed by Northern analysis, is expressed primarily in the kidney and cultured kidney cells regardless of the species (Tate et al., 1992; Yan et al., 1992; Wells and Hediger, 1992; Bertran et al.,1992b). Northern analysis detects rBAT mRNA in both the cortex and medulla of the kidney. In situ hybridization localized the expression of rBAT mRNA in rat kidney primarily to the S3 portion of the proximal tubule (Kanai et al., 1992). Low level expression is also seen in the S1 and S2 segments of the tubule. This localization is consistent with the role of rBAT in amino acid absorption in the kidney, as microperfusion experiments of the proximal straight tubule (S1 and S2) have identified high-affinity cysteine transport in this area (Voelkl et al., 1982; Schafer et al., 1984). High level expression is also seen in all segments of the small intestine (duodenum, jejunum, and ileum). A related transcript also appears in the brain and heart but at slightly larger mRNA sizes (Yan et al., 1992). The identity and role of these related transcripts is unclear.

rBAT protein has been detected by polyclonal antibodies as a 84 to 87 kDa species in epithelial tissue from rat kidney and small intestine (Mosckovitz et al., 1993). Immunohistochemistry and immunoelectronmicroscopy have more discretely localized the rBAT protein to the epithelial membrane lining the rat kidney proximal tubule and to rat jejunal microvilli (Pickel et al., 1993; Furriols et al., 1993).

Functional Analysis of rBAT

Electrophysiological analysis of rBAT injected oocytes has provided some interesting insight into the function of this protein. Two independent laboratories demonstrated the rBAT cRNA mediated electrogenic uptake of both neutral and cationic amino acids (Busch et al., 1994; Coady et al., 1994). Most interesting, however, was the observation that neutral amino acid uptake mediated by rBAT resulted in the generation of outwardly-directed currents, a process expected to be electroneutral with simply the uptake of uncharged substrate. The authors found that preloading of the oocytes with cationic amino acids increased the uptake of neutral amino acids and vise versa. However, significant inhibition of uptake was seen when the preloaded amino acid had a similar charge as the transported substrate. These data are consistent with a model of "trans-stimulation" of uptake where the uptake of charged amino acids is enhanced by the outward flux of neutral amino acids and vise versa.

Role of rBAT in Cystinuria

The location of rBAT and its role in cysteine uptake in the kidney made this protein a likely candidate for cystinuria. This autosomal recessive genetic disease is characterized by high levels of cystine and cationic amino acids in the urine of these patients (Segal and Thier, 1989). The high levels of cystine cause deposits in the urinary tract and lead to infection and renal insufficiency. Investigators suggested a likely cause of this disease was aberrant reabsorption of cystine and cationic amino acids in the kidney and small intestine (Rosenberg et al., 1969). Sequencing of rBAT mRNAs expressed from several patients, and subsequent sequencing of genomic DNA, revealed six point mutations in the rBAT gene specific to cystinuria and represented 30% of the cystinuric patients analyzed (Calonge et al., 1994). The most commonly occurring mutation was the substitution of threonine for methionine at position 467.

This mutation resulted in the decrease of nearly 80% in the transport activity of rBAT expressed in oocytes for all substrates tested (cystine, arginine, leucine). Additional support for the identification of rBAT in cystinuria was provided by linkage analysis which localized the cystinuria gene to chromosome 2P (Pras et al., 1994), the location of rBAT (Lee et al., 1993; Yan et al., 1994).

CHAPTER 2
MATERIALS AND METHODS

This chapter describes the general methods and protocols used throughout this study. Some methods used in Chapters 4 and 6 differ considerably from the methods utilized in the majority of this study described in this chapter. As such, these methods will be described in the respective chapters in which they appear.

Materials

L-arginine [2,3,-^{3}H] and L-glutamic acid [2,3,-^{3}H] were obtained from DuPont New England Nuclear (Boston, MA). Deoxycytidine 5'-[α-^{32}P] triphosphate triethylammonium salt was obtained from Amersham (Arlington Heights, IL). 2-(methylamino) isobutyric acid, [2-methyl-^{3}H], was obtained from ARC (St. Louis, MO). Nitrocellulose filters (0.45 μm) were used for transport assays (Millipore, Bedford, MA). All other reagents and chemicals were of the highest quality available and were obtained from either Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). The MCAT1 (System y^+) cDNA insert was a generous gift from Dr. James Cunningham at the Brigham and Women's Hospital (Boston, MA). The rat EAAC1 cDNA was cloned from a rat hippocampal library as described in Chapter 4. The cathepsin B cDNA was a generous gift of Dr. Harry Nick at the University of Florida (Gainesville, FL). Timed pregnant Sprague-Dawley rat dams were obtained from Zivic-Miller Company (Zellienople, PA).

Methods

Animal feeding. Animals were housed in a temperature-controlled room with twelve hour light/dark cycles and given water *ad libitum*. Animals in gestational studies were given food similar in composition to the control diet, described below, *ad libitum*. Animals in the maternal protein restriction study were weight-matched and separated into either control (C) or low-protein (LP) groups. Both the control diet (19.3% protein, 10.0% fat, 4.3% fiber, 60.6% carbohydrate) and the low-protein diet (4.6% protein, 10.0% fat, 5.7% fiber, 75.3% carbohydrate) were prepared by Purina Mills (St. Louis, MO), and the diets were made isocaloric with the addition of sucrose. In order to ensure that the LP and C groups consumed equal calories throughout the study, LP and C animals were approximately weight matched and subsequently pair-fed from days 6 to 19 gestation. The LP animals were given the low-protein diet *ad libitum,* and the amount of food consumed by each animal each day was recorded. The C animals were then each given the control diet equal in weight to the amount of food consumed by the pair-matched LP animal the previous day. This feeding protocol continued until sacrifice on day 20 gestation. These studies were approved by the Laboratory Animal Medical Ethics Committee of the University of Florida.

Apical and basal membrane vesicle isolation. At day 20 gestation, the dams were sacrificed and placentas removed and weighed. Isolation of rat apical membrane vesicles was performed by a modification of a human preparation (Booth et al., 1980). Minced and washed placental villous tissue from 4 to 8 litters was incubated for 1 hour at 4°C and then passed through 210 µm nylon mesh. All steps were performed at 4°C unless otherwise noted. The filtrate was centrifuged at 800 x g for 10 min. The pellet was discarded and the supernatant centrifuged at 10,000 x g for 10 min. The pellet was again discarded. Crude apical membranes were then obtained by centrifugation at 150,000 x g for 25 min, resuspension in Tris-mannitol buffer (300 mM mannitol, 2 mM Tris-base, pH 7.0), and vesicle formation induced by 12 strokes in a glass homogenizer with Teflon pestle. An equal volume of Tris-mannitol buffer was added to the homogenate and $MgCl_2$ added to a final concentration of 10 mM. An additional 12 stroke homogenization was performed, and the membranes incubated for 10 min. The solution was then centrifuged at 2,200 x g for 12 min and the pellet discarded. The supernatant was centrifuged at 150,000 x g for 25 min to pellet the apical-enriched membrane vesicles. The membranes were then resuspended in HEPES-sucrose buffer (300 mM sucrose, 10 mM HEPES-Tris-base, pH 7.4). An aliquot of the vesicle preparation was used immediately for total protein and marker enzyme analysis and the remainder stored frozen in liquid nitrogen until use. Storage for several months was shown not to affect transport activity.

Basal membrane vesicles were prepared utilizing the tissue remaining on the 210 μm nylon mesh filter from the apical membrane vesicle preparation. This material was washed on the filter three times with ice-cold PBS and then three times with 50 mM Tris-HCl (pH 6.9). The membranes were then removed from the filter by gentle scraping, resuspended in 50 mM Tris-HCl (pH 6.9), and sonicated. The membranes were then re-filtered and washed 3 times with 5 mM Tris-HCl (pH 6.9). The membranes were then incubated with stirring for 30 min, filtered and washed. The washed membranes were then added to an equal volume of Tris-sucrose-EDTA buffer (300 mM sucrose, 2 mM Tris-base, 10 mM NaEDTA, pH 6.9). After an additional incubation at room temperature for 10 min, the solution was again filtered through the 210 μm nylon mesh. The membranes were added to 10 mM EDTA, incubated at room temperature for another 20 min, then sonicated for 20 sec. The suspension was filtered through 6-ply gauze, centrifuged at 4,000 x g for 10 min, and then at 10,000 x g for 20 min. The pellet was discarded, and membrane vesicles pelleted by centrifugation at 150,000 x g for 25 min. The final basal membrane pellet was resuspended in HEPES-sucrose buffer and treated as discussed above for the apical membrane vesicles. Both vesicle preparations were analyzed for total membrane protein as determined by the Lowry method using bovine serum albumin as a standard (Lowry, 1951). Subsequent analysis of vesicle preparations included assays for dihydroalprenolol (DHA) binding, a marker for the basal membrane (Kelley et al., 1983), and alkaline phosphatase, a marker enzyme for the apical membrane (Bowers and McComb, 1966; Glazier et al., 1993).

Vesicle transport assay. Timed uptakes of radiolabelled amino acids were measured by a nitrocellulose filter assay described previously (Novak et al., 1989). In brief, uptakes were measured at 37°C and performed in uptake buffer (10 mM HEPES-KOH, pH 7.5, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$) with either 125 mM Na^+ or K^+ thiocyanate (SCN). All uptake solutions were adjusted to isoosmolarity (310 mosm) with the addition of sucrose. Radiolabelled substrate was then added in tracer amounts to unlabelled substrate to achieve 5-50 μM final concentration, except where otherwise noted, and where indicated, unlabelled amino acid inhibitors were added to the uptake media at the concentrations noted. In each assay, a 20 μl aliquot of vesicle preparation corresponding to 20 - 70 μg protein was incubated at 37°C for 2 min. After temperature equilibration of the membranes, 80 μl of uptake solution described above was added, and the mixture was immediately vortexed. Transport was terminated by the addition of 3 ml of ice-cold stop solution (100 mM NaSCN or KSCN, 10 mM HEPES-Tris pH 7.4, 100 mM sucrose). The entire mixture was then rapidly passed over a 0.45 μm nitrocellulose filter and subjected to vacuum filtration. The uptake tube was washed with an additional 3 ml aliquot of ice-cold stop buffer, and this was poured over the filter as well. The filter was washed two additional times with 3 ml stop buffer and then placed in 4.5 ml Scintiverse Bio-HP scintillation cocktail (Fisher Scientific, Pittsburgh, PA) to determine the bound radioactivity. Membrane binding of substrate was assessed by adding ice-cold stop solution to the radiolabelled substrate prior to mixing with membranes and subsequent filtration. These "blank" values were subtracted from each assay. The uptake velocities are reported as substrate transported$\cdot$mg^{-1} protein$\cdot$unit^{-1} time and are given as the averages ± SEM for 3 - 4 assays.

Fetal hepatocyte isolation. Fetal hepatocytes were isolated by the method of Leffert et al. (1979). Timed-pregnant rat dams were sacrificed on the appropriate day gestation, and the fetuses were removed to 37°C perfusion buffer (25 mM Na_2HPO_4, 3 mM KCl, 119 mM NaCl, 11 mM glucose, 1 mg/ml BSA, pH 7.4). Fetal livers were removed by dissection and placed in sterile 37°C perfusion buffer until all livers were isolated. The buffer was aspirated and replaced with 1 mg/ml collagenase (Sigma, Type I) in perfusion buffer and incubated for 15 minutes at 37°C with vigorious stirring. Dispersed cells were aspirated and placed into 50 ml 37°C arginine-free minimal essential medium (Arg$^-$-MEM) supplemented with 4% fetal bovine serum (FBS). The cells were pelleted by centrifugation at 128 x g for 2 minutes. The medium was then removed and replaced with 25 ml of same. To the remainder of the liver tissue, an additional aliquot of 1 mg/ml collagenase was added and incubated again at 37°C. The cells from both incubations were pooled and washed five times in Arg$^-$-MEM/4% FBS. Visual inspection of the pellet for the absence of red blood cells was performed, and if contamination by these cells was suspected, the cells were washed additionally in the same manner until removal of contaminants. Cells were counted and viability estimated by exclusion of trypan blue. Hepatocytes isolated by this procedure had greater than 95% viability. The cells were then plated on culture plates pre-coated with 0.02 mg/ml collagen (Sigma, Type III) at a density of 500,000 cells per well of a 24-well cluster tray (Costar)(Kilberg, 1989). Cells were cultured for only 24 hours to allow the cells to attach as it has been demonstrated that after this time in culture, the hepatocyte amino acid transporter complement is significantly altered (Closs et al., 1993).

Whole cell transport assay. Amino acid uptake of adherent hepatocytes was measured by the cluster tray method of Gazzola et al. (1981) with modifications by our laboratory (Kilberg et al., 1989). Hepatocytes were plated on 24-well Costar trays as described above. To deplete the intracellular pools of amino acids to minimize trans-effects on transport (Kilberg et al., 1989), cells were incubated at 37°C for 30 minutes in Na^+-Krebs-Ringer phosphate (Na^+KRP)(119 mM NaCl, 25 mM Na_2HPO_4, 5.9 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $KHCO_3$, 5.6 mM glucose, 0.5 mM $CaCl_2$, pH 7.4). Radiolabelled substrate and the appropriate inhibitors, were then added to 0.25 ml of Na^+KRP, or to measure Na^+-independent uptake, to 0.25 ml choline-Krebs-Ringer phosphate (cholKRP)(corresponding Na^+-salts substituted with choline chloride and choline phosphate at the same concentration, pH 7.4). After transport, cells were washed 4 times with 3 ml ice-cold cholKRP and subsequently air-dried. Cells were then solubilized in 0.2 ml 0.2 N NaOH/0.2% SDS for 15 minutes. A 0.1 ml aliquot was added to vials with 5 ml scintillation cocktail (Scintiverse, Fisher Scientific) to determine radioactivity. The remaining 0.1 ml was used for estimation of total protein as determined by the method of Lowry (1951).

SDS-PAGE and immunoblotting. SDS-PAGE (7.5%) was performed by the method of Laemmli (1970). Ater electrophoresis, the proteins were electrotransferred to a 0.45 μM nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Primary antibody was diluted 1:250-1:1000 in blocking buffer (5% non-fat dry milk in 10 mM Tris-Cl, pH 7.5, 150 mM NaCl), and incubated with the blot for 1 h at room temperature with agitation. Horseradish peroxidase-conjugated Protein A was used at 1:20,000 dilution in blocking buffer for detection of the immunoreactive band by visualization with the Enhanced Chemiluminescence Kit (Amersham, Arlington Heights, IL). Densitometric analysis was performed and presented as relative absorbance units per microgram of protein.

RNA Isolation and Northern Analysis. Dams were sacrificed, and for each isolation, one gram of placental tissue was snap-frozen and ground to a powder in a mortar and pestle under liquid nitrogen. The placental powder was then added to denaturing solution (4 M guanidinium thiocyanate, 0.5% N-lauryl sarcosine, 25 mM sodium citrate pH 7.0, 100 mM ß-mercaptoethanol) to inhibit RNAse activity. The solution was homogenized with 10 strokes in a glass homogenizer with a motor-driven Teflon pestle. Total RNA was isolated by the method of Chomczynski and Sacchi (1987), and poly A^+ selected mRNA was isolated from 500 μg total RNA using the Poly ATract System (Promega, Madison, WI). Equal amounts of poly A^+ selected mRNA were loaded per lane (3 μg) and subjected to 1% agarose gel electrophoresis in the presence of 0.02 M formaldehyde. The RNA was capillary transferred to nylon membrane and hybridized with ^{32}P-labeled cDNA probe prepared by random priming extension (GibcoBRL, Gaithersburg, MD). The resulting autoradiogram was quantified by densitometry. To correct for loading differences, the levels of mRNA were normalized to the constituatively expressed cathepsin B mRNA. Experiments having loading differences of more than 10% per lane were not used to quantify mRNA content. Preliminary experiments were performed to ensure that the autoradiographic exposures were within the linear range of the film and densitometer.

CHAPTER 3
DEVELOPMENTAL REGULATION OF CATIONIC AMINO ACID TRANSPORT IN RAT PLACENTA

Introduction

The placenta serves as the functional interface between the maternal and fetal circulations and is capable of concentrative amino acid delivery to the fetus via transporters in both the apical (maternal-facing) and basal (fetal-facing) membranes of the syncytiotrophoblast (Smith et al., 1992). Of all the amino acids in humans, those demonstrating the highest fetal to maternal serum ratios are the cationic amino acids arginine and ornithine (Economides et al., 1989). Additionally, arginine is unique in that the fetomaternal serum ratio increases with increasing gestational age (Bernardini et al., 1991). As the nutritional needs of the developing fetus change, it is not unreasonable to suspect that the placental delivery of nutrients, and therefore the transport systems responsible for their delivery from the maternal circulation, change in response to those needs.

Arginine plasma membrane transport in mammalian cells is mediated by at least four kinetically distinct transport systems. With the exception of the liver, in the adult mammal, the primary route of cationic amino acid uptake is via the Na^+-independent, high affinity System y^+ (White, 1985). However, the normal adult liver lacks System y^+ and instead contains a low-affinity, liver-specific transporter with similar substrate specificity to System y^+ (Kim et al., 1991). The corresponding cDNAs (mCAT1 and mCAT2a) have been isolated (Kim et al., 1991; Closs et al., 1993). Two additional transport systems, first described in mouse blastocysts, transport not only cationic amino acids but small neutral amino acids as well. These activities are the Na^+-dependent System $B^{o,+}$ and the Na^+-independent System $b^{o,+}$ (Van Winkle et al., 1985; Van Winkle et al., 1988). Another Na^+-independent transporter of both cationic and neutral amino acids described in the erythrocyte, with substrate specificity and kinetic properties very similar to System $b^{o,+}$, is System y^+L (Devés et al., 1992). The primary kinetic difference between these two systems is the inhibition by neutral amino acids. In System $b^{o,+}$, neutral amino acids inhibit the uptake of cationic amino acids in the presence and absence of Na^+ (Van Winkle et al., 1988) while System y^+L requires the presence of Na^+ for neutral amino acid inhibition of cationic uptake (Devés et al., 1992). However, since System y^+ has been shown to interact with neutral amino acids in the presence of Na^+ (Christensen and Handlogten, 1969), the identity of System y^+L remains ambiguous. In addition to these activities, the intestinal brush border has both Na^+-dependent and Na^+-independent cationic amino acid transport systems which resemble Systems $B^{o,+}$ and $b^{o,+}$ respectively, with a somewhat narrower substrate specificity (Cassano et al., 1983; Harvey et al., 1993; Wolfram et al., 1984). Injection of intestinal mRNA into oocytes induced System $b^{o,+}$ activity, the primary route of endogenous transport in *Xenopus* oocytes for neutral and cationic amino acids (Campa and Kilberg, 1991), by the synthesis of a Type II membrane protein (rBAT) with a structure that is dissimilar to those of other known transporters (Magagnin et al., 1992).

The trophoblast of human placenta has been well characterized as to the complement of nutrient transporters by several laboratories using many different methods (Yudilevich and Sweiry, 1985; Smith et al., 1992). This subject has been reviewed in the Introduction. However, few reports exist on the kinetic discrimination of the cationic amino acid transport systems in the placenta.

Wheeler and Yudelivich (1989) described the transplacental flux of lysine and alanine in an in situ dually-perfused placental system in the guinea pig. Saturable transport systems for lysine were identified on both the apical and basal membranes, with a significantly higher flux at the maternal (apical) membrane. Additionally, an increased efflux of lysine was detected on the basal membrane. These data are consistent with the presence of an increased lysine transporter number on the apical membrane for the extraction of this nutrient from the maternal circulation and delivery to the fetus. This lysine uptake and efflux was inhibited strongly by other cationic amino acids (arginine, ornithine) and more weakly by histidine, an amino acid with about 0.1% cationic species at physiologic pH (Mann et al., 1984). While the neutral amino acid alanine significantly inhibited the uptake of lysine consistent with either System $y^{+}L$ or y^{+} in the presence of Na^{+} (Christensen and Handlogten, 1969), the reciprocal inhibition was not noted leaving system assignment difficult.

In purified membrane preparations from term human placentas, cationic amino acid uptake was seen in both apical and basal membrane vesicles (Kudo et al., 1987; Kudo and Boyd, 1990). However, the extremely high inhibitor concentrations used (30-50 mM), 100-fold to 10,000-fold over the published K_m for these transport systems (White, 1985), makes discrimination of transport systems difficult. It should be noted that in this study, inhibitor concentrations of this magnitude show noncompetitive inhibition of nearly all transport systems.

Furesz and coworkers (1991), examined cationic amino acid uptake in basal membrane vesicles derived from human placenta. Two systems were identified in basal membrane vesicles with similar affinity for both arginine and lysine (100-200 μM). Inhibition of each system by neutral amino acids in the absence of Na^{+}, gave kinetic results consistent with the presence of Systems y^{+} and $b^{o,+}$. Subsequently, the same group of investigators described cationic amino acid in human apical membrane vesicles (Furesz et al., 1995). Similar results to that of the basal membrane were described with the exception of neutral amino acid inhibition of cationic amino acid uptake. Inhibition of the apical cationic transport systems by neutral amino acids required the presence of Na^+ more consistent with System y^+L than $b^{o,+}$.

There have been no reports of the characterization of cationic amino acid transport activity in the rat placenta, and no reports to address the effect of the needs of the developing fetus on this activity with the exception of the manuscript published from the data described in this chapter (Malandro et al., 1994). This chapter describes changes in cationic amino acid transport occurring with increasing gestational age in a rat placental model. Competitive inhibition analysis using L-arginine as a substrate was used to identify the transport systems responsible for cationic amino acid uptake at 14 to 20 days gestation. Vesicle preparations enriched for either apical or basal membranes were used to establish differences in transport activities between plasma membrane domains. The presence of System y^+ was further demonstrated by the analysis of mRNA content throughout gestation.

Results

Characterization of apical and basal membrane vesicle preparations. To ensure that uptake measurements from vesicles isolated from placentas of different gestational ages would not be affected by variations in size, vesicle volumes and diameters were examined at the two age extremes,

Table 3-1. Characterization of apical and basal membrane vesicles from rat placentas of 14 or 20 days gestation.

	14 Day Apical	20 Day Apical	14 Day Basal	20 Day Basal
Vesicle Diameter	0.45	0.46	0.43	0.36
Marker Enzyme Enrichment				
-Alkaline Phosphatase	48.3 ± 23	43.0 ± 12	3.3 ± 1	2.4 ± 1
-DHA Binding	2.7 ± 1	2.7 ± 1	15.1 ± 5	16.3 ± 7

Vesicle diameter was determined by direct sizing by laser-light diffraction analysis (Nicomp Model 270; Particle Sizing Systems Inc., Goleta, CA). Vesicle diameters are given as μm. Marker enzyme data are given as -fold enrichment over homogenate and represent the mean ± standard deviations for at least 6 independent vesicle preparations.

14 and 20 days gestation. For both apical and basal membrane vesicles, volume, as determined by the steady-state distribution of ^{3}H-alanine (Kletzien et al., 1975), did not significantly differ with gestational age in either preparation. Average vesicle diameters estimated by laser-light diffraction analysis (Niven et al., 1990) of each membrane preparation were similar for both gestational ages as well (Table 3-1).

The alkaline phosphatase enrichments of the apical membrane preparation (Table 3-1) were comparable to or greater than those reported by several investigators for human placenta (Alonso-Torre et al., 1992; Karl et al., 1989; Moe and Smith, 1989) and for rat placenta (Glazier et al., 1993). The enrichment of DHA binding seen in the basal membrane preparation was not as high as that reported for the human basal vesicle preparation of Kelley, Smith and King (1983), but was comparable to enrichments reported by other investigators (Bravo et al., 1993, Marin et al., 1990).

Although the basal membrane preparation is contaminated with a small percentage of apical membranes, several lines of evidence exist suggesting a functional difference between the two preparations. First, the Na^+-dependent arginine transport activity clearly present in apical membrane vesicles was not detectable in the basal membrane preparation after 14 days gestation (Figure 3-3). It is not likely that the Na^+-dependent System $B^{o,+}$ was inactivated in the preparation of basal membrane vesicles due to the fact that other Na^+-dependent amino acid transporter systems such as Systems X^-_{AG} and A can be detected in basal membrane vesicles (Chapters 4 and 5, respectively). Second, the different gestational related changes in System y^+ and leucine-sensitive arginine transport in the two preparations suggest a functional separation of the two membrane surfaces (Figure 3-3). The contamination of the basal membrane preparation with apical membranes if anything, would cause an under-estimation the magnitude of these gestational changes.

Characterization of arginine transport systems of the apical and basal membranes. To establish that ^3H-arginine associated with the membrane vesicles was due at least in part to transport and not merely non-specific binding, steady state (10 min) arginine uptake was examined after incubation in buffer containing increasing extravesicular osmolarity (Lever, 1980). The arginine accumulation decreased with increasing extravesicluar osmolarity demonstrating transport into an osmotically active space.

As arginine is a substrate for at least four distinct transport systems, three of which have been described in more than one tissue, we characterized arginine uptake through each of these systems. Low affinity Na^+-independent cationic amino acid transport mediated by mCAT2a is considered liver specific in its distribution (Closs et al., 1993). To confirm the absence of mCAT2a in the placenta, Northern blot analysis was performed with an mCAT2a cDNA, and rtPCR analysis with mCAT2a specific primers. No detectable

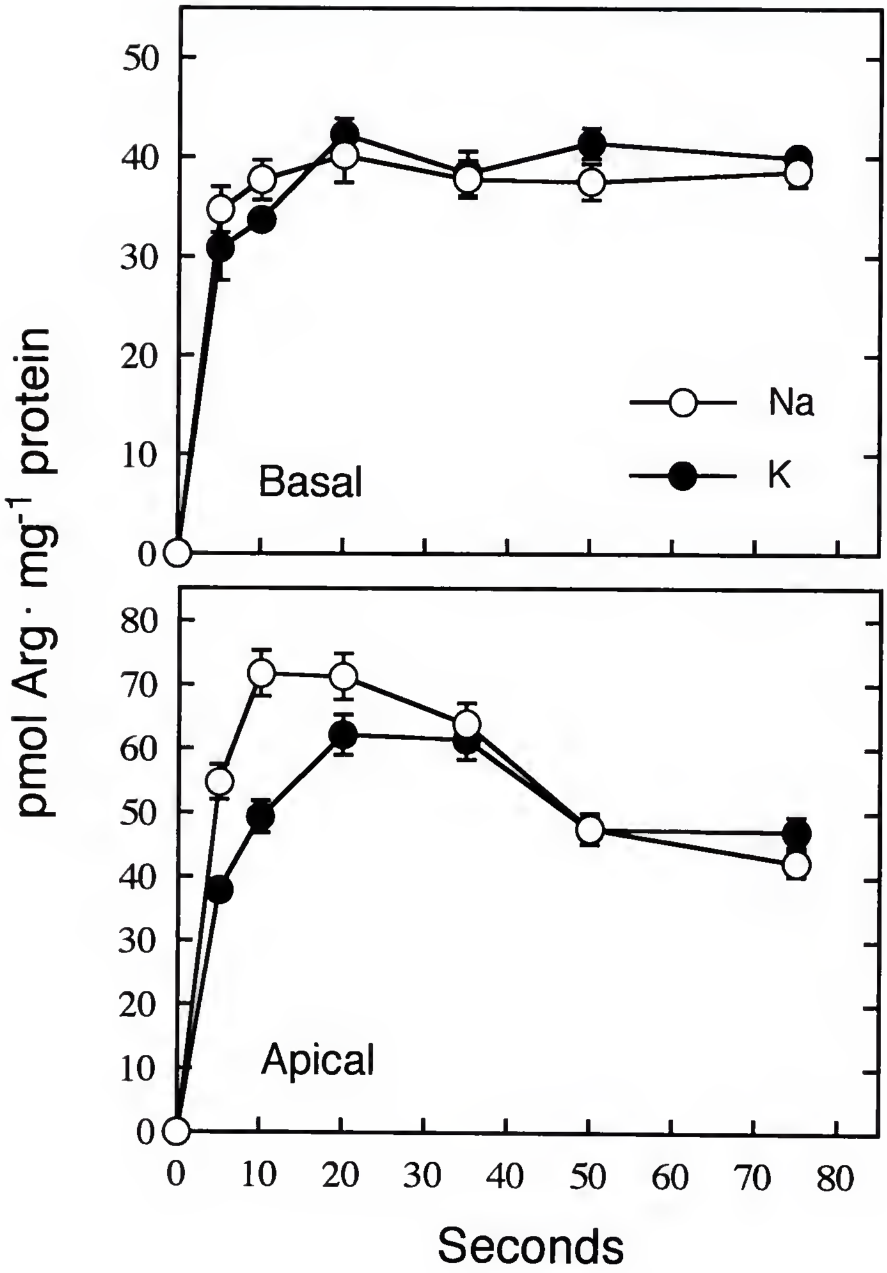


Figure 3-1. Time-course of [^{3}H]-arginine uptake in basal or apical membrane vesicles from placentas of 20 days gestation. Transport of 10 μM [^{3}H]-arginine was performed at 37°C in uptake buffer containing a final concentration of either 100 mM NaSCN (open circles) or 100 mM KSCN (closed circles). Equilibrium points were assayed in both basal (Na^+ = 42 ± 1.9, K^+ = 40 ± 0.6) and apical (Na^+ = 47 ± 0.9, K^+ = 41 ± 2.2) membranes. Values are reported as the means ± standard deviations for triplicate determinations. Where not shown, the standard deviation bars are contained within the symbol.

hybridization or PCR product was observed with either total RNA or poly $(A)^+$ selected mRNA isolated from placentas at 14 through 20 days gestation when total or poly $(A)^+$ mRNA from rat liver was used as a positive control.

Na^+-dependent cationic amino acid transport occurs in mouse blastocysts (Van Winkle et al., 1985), *Xenopus* oocytes (Campa and Kilberg, 1991), and several other cell types (Christensen and Kilberg, 1987; Kilberg et al., 1993) by an activity termed System $B^{o,+}$. This system also is capable of neutral amino acid transport. A time course of total L-arginine uptake (10 μM) in either apical or basal membrane vesicles from placentas of 20 days gestation was examined in the presence of 100 mM NaSCN or KSCN (Figure 3-1). Subsequent experiments showed arginine uptake was approximately linear to 10 sec, thereafter approaching equilibrium. Given the technical difficulties for measuring transport at shorter times, 10 sec uptakes were used to approximate initial rates in all the uptake assays reported for both membrane preparations. The virtual superimposition of the Na^+ and K^+ time course curves in basal membrane vesicles (Figure 3-1) documents the absence of Na^+-dependent arginine uptake and hence, the absence of System $B^{o,+}$ in this preparation. In contrast, as shown in Table 3-2, Na^+-dependent uptake was noted in apical membranes which was nearly completely inhibited by arginine or lysine; the neutral amino acids, homoserine (83%), leucine (56%), and the branched chain analog 2-aminobicyclo [2,2,1] heptane-2-carboxylic acid

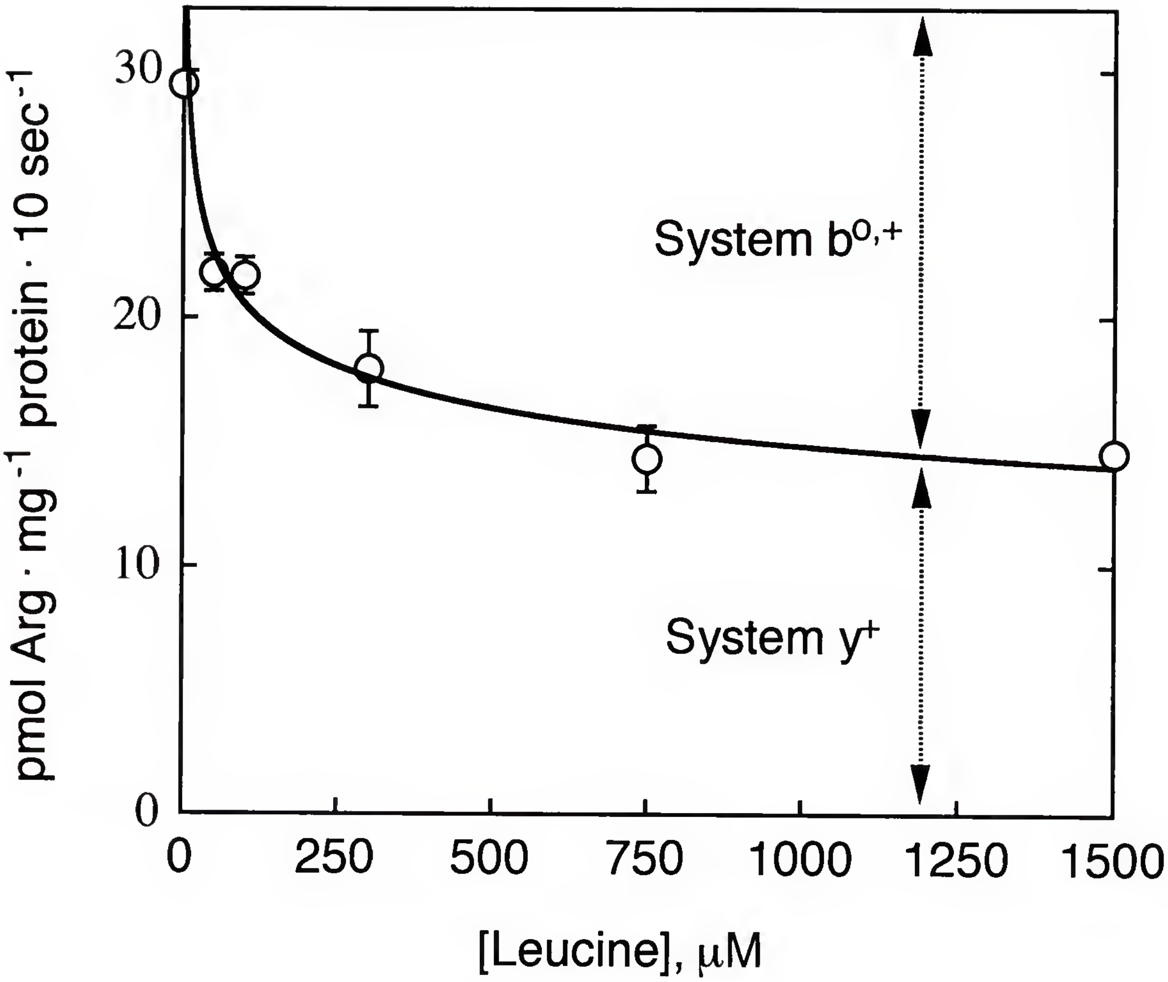


Figure 3-2. Inhibition of Na^+-independent $[^3H]$-arginine uptake with increasing concentrations of leucine in placentas of 20 days gestation. Uptake of 10 μM $[^3H]$-arginine was performed for 10 sec at 37°C in uptake buffer containing a final concentration of 100 mM KSCN and the indicated concentration of leucine. All uptake solutions were adjusted to isoosmolarity (310 mosm) with the addition of sucrose. Values are reported as the means ± standard deviation for triplicate determinations. Where not shown, the standard deviation bars are contained within the symbol.

(BCH)(49%) also showed some inhibition. Each of these amino acids are known substrates for System $B^{o,+}$ in blastocysts (Van Winkle et al., 1985).

The Na^+-independent uptake of cationic amino acids can occur through several transport systems including Systems y^+, $b^{o,+}$ and y^+L. System y^+ is present in most mammalian cells and at physiologic substrate concentrations appears to mediate the transport of cationic amino acids primarily (White, 1985). However, in the presence of Na^+, neutral amino acids at non-physiologic concentrations can competitively inhibit System y^+ (Christensen and Handlogten, 1969). System $b^{o,+}$, first described in mouse blastocysts (Van Winkle et al., 1988) and later in *Xenopus* oocytes (Campa and Kilberg, 1991) and basal membrane vesicles from human placenta (Furesz et al., 1991), accepts both neutral and cationic amino acids. System $b^{o,+}$ activity is typically measured as leucine-inhibitable lysine or arginine transport or vice versa (Van Winkle et al., 1988). Another transport system recently described in human erythrocytes, System y^+L, is similar in properties to System $b^{o,+}$ and can be assayed in a similar fashion (Devés et al., 1992). In the rat apical membrane vesicles prepared from placentas of 20 days gestation, arginine uptake was partially inhibited by the presence of leucine at 50-1500 μM (Figure 3-2). A Dixon plot of the data from a separate experiment measuring arginine uptake (10 - 100 μM) at increasing concentrations of leucine, indicated competitive inhibition with an estimated K_i value of

Table 3-2. Inhibition of Na$^+$-dependent (System B$^{o,+}$) arginine uptake in microvillous membrane vesicles from placentas of 20 days gestation.

	Na$^+$	K$^+$	Na$^+$-dependent	%Inhib
No Inhibitor	60.2 ± 5.1	41.9 ± 2.1	18.3	--
Arginine	3.6 ± 1.0	3.5 ± 1.3	0.1	99
Lysine	2.2 ± 0.7	2.0 ± 0.3	0.2	99
Homoserine	32.9 ± 2.4	29.7 ± 2.5	3.2	83
Leucine	37.0 ± 4.5	29.0 ± 1.2	8.0	56
Proline	30.7 ± 3.1	30.3 ± 1.1	0.4	98
BCH	39.4 ± 0.5	30.0 ± 1.5	9.4	49

The uptake of 10 μM ^{3}H-arginine was assayed for 10 sec at 37°C in either Na$^+$ or K$^+$ -containing buffer as described in Materials and Methods (Chapter 2). Inhibitors were included at a concentration of 2.0 mM, and solutions were brought to isoosmolarity (310 mosm) by the addition of sucrose. The values represent the means (n = 3) ± S.D. in pmol·mg^{-1} protein·10 sec^{-1}. All inhibitors were significantly ($P < 0.005$) different from control (no inhibitor) (BCH = 2-aminobicyclo [2,2,1] heptane-2-carboxylic acid)

Table 3-3. Inhibition of Na$^+$-independent, leucine-sensitive arginine uptake in apical and basal membrane vesicles from placentas of 20 days gestation.

Microvillous

	-Leu	+Leu	Leu-sensitive	%Inhib
No Inhibitor	38.4 ± 1.3	29.1 ± 2.5	9.3	--
Arginine	3.5 ± 1.3	4.1 ± 1.1	--	100
Lysine	2.0 ± 0.3	2.4 ± 0.1	--	100
Homoserine	29.7 ± 2.1	22.5 ± 2.2	7.2	23
Proline	30.3 ± 1.1	20.3 ± 2.0	10.0	0
BCH	30.0 ± 1.5	19.2 ± 2.1	10.8	0

Basal

	-Leu	+Leu	Leu-sensitive	%Inhib
No Inhibitor	54.0 ± 3.1	32.5 ± 1.6	21.5	--
Arginine	8.7 ± 0.5	11.3 ± 1.2	--	100
Lysine	4.5 ± 1.8	6.2 ± 1.5	--	100
Homoserine	31.8 ± 1.1	14.7 ± 2.2	17.1	20
Proline	41.6 ± 5.5	15.4 ± 1.6	26.2	0
BCH	36.7 ± 3.8	13.8 ± 1.5	22.9	0

The uptake of 10 μM ^{3}H-arginine in the presence or absence of 10 mM leucine was assayed for 10 sec at 37°C as described in Materials and Methods. Inhibitors were included at a concentration of 2.0 mM, and solutions were brought to isoosmolarity by the addition of sucrose. The values represent the means (n=3) ± S.D. in pmol·mg^{-1} protein·10 sec^{-1}. (BCH = 2-aminobicyclo [2,2,1] heptane-2-carboxylic acid) approximately 500 μM. Thus, the level of leucine used as an inhibitor in subsequent assays, at least 2.5 mM, should be adequate to block most if not all of the leucine-sensitive component of arginine uptake. Analysis of the leucine-sensitive arginine uptake in both the basal and apical membranes (Table 3-3), showed complete inhibition by the cationic amino acids arginine and lysine, and partial inhibition by the neutral amino acid homoserine (54% basal and 76% apical). Additionally, no significant inhibition was seen with BCH or proline, previously shown to be poor substrates for System $b^{o,+}$ as well as System y^+L (Devés et al., 1992; Van Winkle et al., 1988).

Even at high concentrations of leucine (Figure 3-2), not all arginine uptake was inhibited indicating the presence of a leucine-insensitive component. To further characterize this portion of arginine uptake, inhibitor studies were performed (Table 3-4). Only a small amount of inhibition was seen with homoserine, proline, and BCH. A similar degree of inhibition with these amino acids was noted by other investigators in basal membrane vesicles prepared from human term placentas (Furesz et al., 1991). In contrast, significant inhibition was noted with arginine (91% apical and 73% basal) and lysine (92% apical and 86% basal). Inhibition of leucine-insensitive arginine uptake by homoserine in the presence of Na^+ was also seen (87% apical and 92% basal). These data indicate that the Na^+-independent leucine-insensitive component of arginine uptake is mediated

Table 3-4. Inhibition of Na^+-independent, leucine-insensitive (System y^+) arginine uptake in apical and basal membrane vesicles from placentas of 20 days gestation.

	Apical	Basal
	%INHIBITION	%INHIBITION
No Inhibitor	--	--
Arginine	91	73
Lysine	92	86
Homoserine	23	16
Proline	12	12
BCH	19	17

Uptake of 10 μM ^{3}H-arginine was assayed in the presence of 10 mM leucine for 10 sec at 37°C as described in Materials and Methods. Inhibitors were included at a concentration of 2.0 mM, and solutions were brought to isoosmolarity by the addition of sucrose. The results are the average of at least three assays, and the standard deviations were typically less than 10%. The control values (no inhibitor) for apical and basal membranes were 29.1 ± 2.5 and 32.5 ± 1.6 $pmol \cdot mg^{-1}$ $protein \cdot 10\ sec^{-1}$, respectively. (BCH = 2-aminobicyclo [2,2,1] heptane-2-carboxylic acid) by a cationic amino acid-preferring system similar to System y^+.

Ontogeny of cationic amino acid transport systems with increasing gestational age. As the fetal to maternal serum ratio for arginine increases with increasing placental development (Bernardini et al., 1991, Economides et al., 1989), the contribution of each cationic amino acid transport system to arginine uptake at 14 to 20 days gestation was examined. The transport activities, defined as described above, were examined in both the apical and basal membranes (Figure 3-3). System $B^{o,+}$ activity on the basal membrane was detectable at 14 days but, in agreement with Figure 3-1, was absent from 16 to 20 days gestation. In contrast, System $B^{o,+}$-mediated arginine uptake in the apical membrane increased by 4-fold, from 3.0 ± 0.2 to 12 ± 1.5 $pmol \cdot mg^{-1}$ $protein \cdot 10$ sec^{-1} over the same gestational period. The Na^+-independent, leucine-sensitive component of arginine uptake increased with increasing gestational age from 14 to 20 days in both the apical (0.8 ± 0.4 to 6.6 ± 2.0 $pmol \cdot mg^{-1}$ $protein \cdot 10$ sec^{-1}) and basal (1.8 ± 0.3 to 15 ± 2.6 $pmol \cdot mg^{-1}$ $protein \cdot 10$ sec^{-1}) membrane. The Na^+-independent System y^+ component of arginine uptake increased from 14 to 16 days gestation in the basal membrane (2.3 ± 0.3 to 8.2 ± 1.0 $pmol \cdot mg^{-1}$ $protein \cdot 10$ sec^{-1}) and then remained fairly constant throughout the gestational period. A steady increase of nearly 10-fold in System y^+ activity was seen in the apical membrane over the same

Figure 3-3. Ontogeny of cationic amino acid transport systems in microvillous and basal membrane vesicles. Cross-hatched bars represent uptake by microvillous membrane vesicles and the filled bars, represent uptake by basal membrane vesicles. A) Na^+-dependent uptake of 10 μM [^{3}H]-arginine (System $B^{o,+}$) was assayed for 10 sec at 37°C. Values were calculated from the difference in uptake in 100 mM NaSCN and KSCN buffers. B) The Na^+-independent, leucine-sensitive uptake of 10 μM [^{3}H]-arginine. Values were calculated from the difference in uptake in KSCN buffer and KSCN buffer containing 2.5 mM leucine. C) The Na^+-independent, leucine-insensitive uptake of 10 μM [^{3}H]-arginine (System y^+). Values were calculated from the difference in uptake in KSCN buffer containing 10 mM leucine and KSCN buffer containing 10 mM unlabelled arginine. Values represent the means ± standard deviations for triplicate determinations from 2 independent preparations at each day gestation.

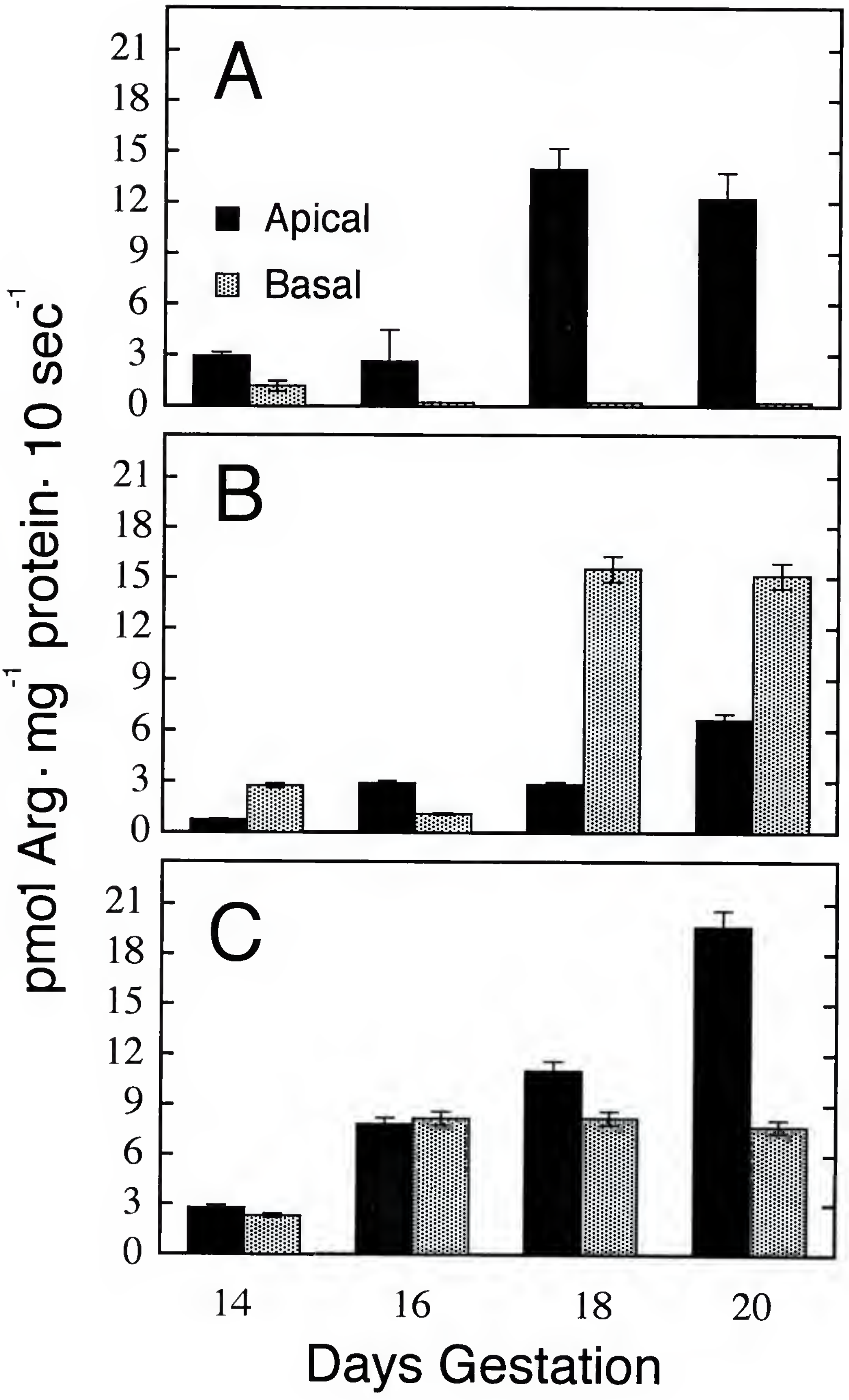
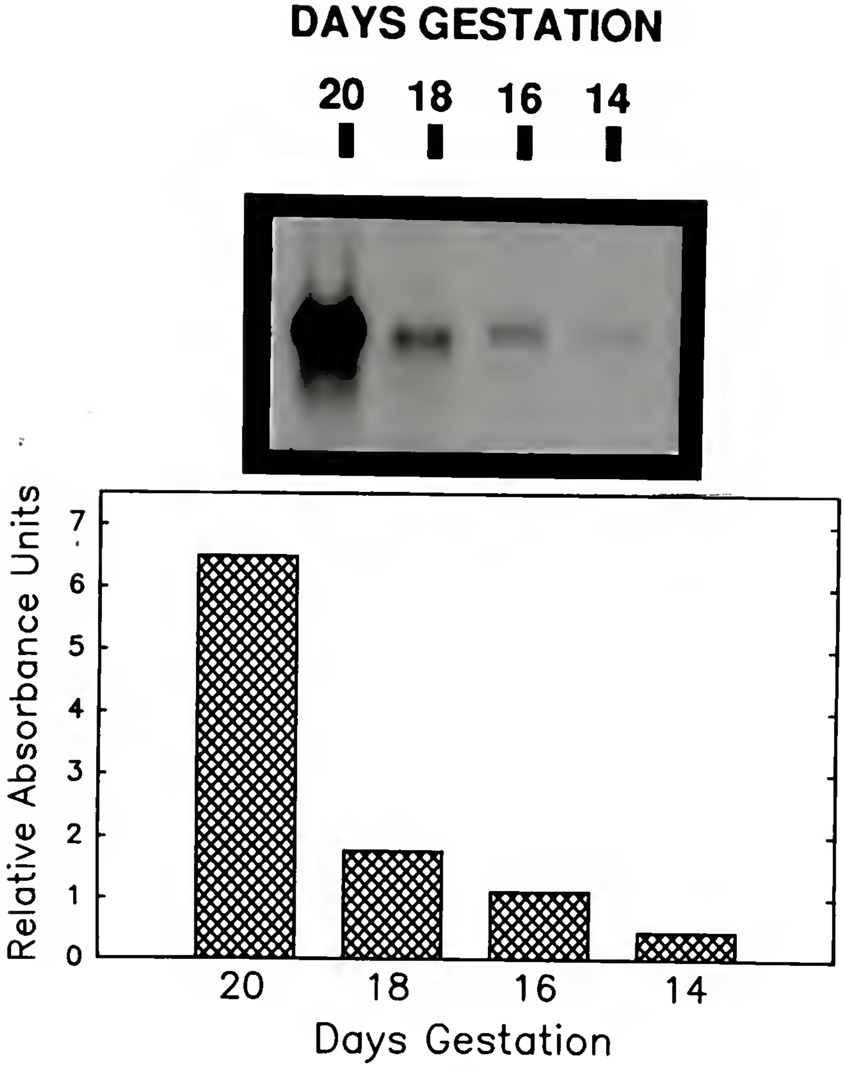

A
B
C
Apical
Basal
0
3
6
9
12
15
18
21
pmol Arg · mg^{-1} protein · 10 sec^{-1}
14
16
18
20
Days Gestation

Figure 3-4. Northern analysis of System y^+ mRNA from placentas of increasing gestational age. Total placental RNA (30 μg per lane) was separated by 1% agarose gel electrophoresis and transferred to nylon membrane. The filter was hybridized with ^{32}P-labelled System y^+ cDNA probe and washed at 65°C (375 mM NaCl, 40 mM Na_2HPO_4, pH 7.2, 1 mM EDTA, 1% SDS). A single mRNA species was detected at 7.4 - 7.9 Kb in each sample (Top). Densitometric analysis of the autoradiogram was performed (Bottom), and adjusted for any loading differences (< 10%) by normalization to the 28S ribosomal RNA, quantified by densitometry of the photographic negative of the ethidum bromide stained gel. Values are representative of four independent experiments and expressed in relative absorbance units.


DAYS GESTATION
20
18
16
14
Relative Absorbance Units
0
1
2
3
4
5
6
7
20
18
16
14
Days Gestation

gestational period (2.8 ± 0.3 to 20.0 ± 2.0 pmol·mg^{-1} protein·10 sec^{-1}).

Northern analysis of System y^+ mRNA levels. A mouse cDNA probe (mCAT1) for the System y^+ transporter (Kim et al., 1991) was used to correlate the level of activity with the cellular mRNA level (Figure 3-4). Consistent with the mCAT1 mRNA size in other tissues and species, an mRNA species of approximately 7.4 - 7.9 kb was detected in total RNA isolated from placentas of 14 to 20 days gestation. No molecular probes have been described for either System $B^{o,+}$ or $b^{o,+}$. Densitometric analysis revealed a significant increase in the steady-state content of System y^+ mRNA over the gestational period. These data parallel the observed increase in arginine transport via System y^+ in both the apical and basal membranes (Figure 3-3).

Discussion

The rat provides an excellent model system for the study of amino acid transport in the developing placenta. The rat has a hemochorial placenta similar to the human, the major difference being an additional two trophoblast layers between the maternal and fetal circulations. Alkaline phosphatase has been shown by cytochemistry to be localized to the apical membrane of the human trophoblast and to the apical membrane of rat trophoblast layer II (Glazier et al., 1993). Rat trophoblast layer I is fenestrated with large gaps allowing for the nearly unrestricted passage of large molecules, such as horseradish peroxidase, injected into the maternal circulation (Metz et al., 1978). These molecules are nearly totally excluded by trophoblast layer II. Therefore, it is likely that the first functional barrier encountered by a solute in the maternal circulation is trophoblast layer II, and as such, the apical membrane preparation from the rat placenta is enriched for a membrane population analogous to that of the human trophoblast apical membrane. We cannot definitively assign the ß-adrenergic receptor, and thus DHA binding, to a specific basal membrane of trophoblast layers II or III.

In the original description of the basal membrane preparation from human placenta (Kelley et al., 1983), the association of the basal membrane with the basal lamina is critically important, and it is this interaction exclusively that prevents the removal of this membrane under conditions necessary for the removal of the apical membrane and the cytoplasmic contents of the trophoblast. The adjustment of the EDTA concentration and the additional sonication then are responsible for the stripping of this membrane from the basal lamina. Only the basal membrane of trophoblast layer III is in association with the basal lamina in the rat trophoblast. As both layers II and III are syncytial, the conditions described for the removal of the apical membrane and syncytial cytoplasm in the human basal membrane preparation should remove these membranes as well leaving the basal membrane of layer III attached to the basal lamina. As these observations have never been tested, the basal membrane preparation could have arisen from either trophoblast layers II or III. We would suggest that even if the membrane preparation was to arise from the basal membrane of layers II or III, this would still represent a functional basal membrane barrier between the apical membrane and the fetal circulation. It should be pointed out that the transporters we have assigned to the rat basal membrane also have been assigned to the basal membrane of the human trophoblast (Furesz et al., 1991) lending even more credence to the analogy between the human basal membrane and our basal membrane preparation. In addition, Ca^{2+}ATPase localized to the basal membrane of the human trophoblast has been localized by immunohistochemistry to the basal membrane of trophoblast layer III in the rat placenta (Borke et al., 1989). These data also support the functional similarity between the human trophoblast basal membrane and the rat basal membrane of trophoblast layer III.

The current study demonstrates that cationic amino acid uptake in the rat placental syncytiotrophoblast is mediated by an asymmetric distribution of both Na^+-dependent and Na^+-independent transport systems in the apical and basal membranes resulting in the net flux of amino acids from the maternal to fetal circulations. The uptake of amino acids from the maternal circulation into the syncytial cytoplasm takes place at the apical membrane, in direct apposition to the maternal blood supply. Our data show that System $B^{o,+}$, a known secondary-active Na^+-dependent transporter of cationic as well as neutral amino acids, to be present within this membrane domain. By virtue of the inwardly directed Na^+ gradient *in vivo*, it contributes to the concentrative transfer of cationic amino acids across the trophoblast. The concentrated levels of arginine and lysine in the human trophoblast, 0.32 and 0.51 mM respectively, compared to the maternal serum levels of these amino acids, 0.043 and 0.10 mM respectively (Economides et al., 1989), would support this hypothesis. However, both Systems y^+ and the neutral amino acid-inhibitable system (System $b^{o,+}$), are also present on the apical membrane. These Na+-independent non-concentrative systems presumably play an important role in the regulation of the syncytial cytoplasmic levels of these amino acids by permitting transfer in the reverse direction if necessary.

The basal membrane of the syncytiotrophoblast, in contrast to the apical membrane, has little or no significant Na^+-dependent cationic amino acid uptake. The presence of System $B^{o,+}$ on the fetal-facing membrane could result in the non-specific and perhaps detrimental clearance of neutral as well as cationic amino acids from the fetal circulation. In contrast, the high expression of both Na^+-independent systems on the basal surface is important to mediate the transfer of amino acids to the fetus by facilitative transport down their concentration gradient. However, when net flux of cationic amino acids from maternal to fetal circulation is considered, the role of membrane potential (Bussolati et al., 1987), as well as multiple transport systems with overlapping substrate specificities must be evaluated and better understood.

As was previously mentioned, the fetal to maternal plasma ratio of arginine increases with increasing gestational age (Bernardini et al., 1991). Our data demonstrate that this may be due in part to the concurrent developmental changes in the transporter complement of the apical and basal trophoblast membranes. During 14 to 20 days gestation there is a significant increase in rate of secondary-active transport by System $B^{o,+}$ on the apical membrane. This probably participates in the increased clearance of cationic amino acids from the maternal circulation; the subsequent release of these into the fetal circulation would occur by one or both of the Na^+-independent cationic amino acid transporters on the basal membrane, both of which increase with increasing gestational age as well.

With respect to the placental transport of amino acids, previous studies have included only activity measurements to investigate changes during development. Our study is the first to use a cDNA probe to provide further evidence for the existence and regulation of a particular placental amino acid transporter. Northern analysis demonstrates that the developmental regulation of System y^+ activity is accompanied by an increase in the steady-state mRNA level for the transporter as well. Thus, either transcriptional control or mRNA stabilization must play a role in regulating cationic amino acid transport during placental development.

Characterization of the rat placental amino acid transporters with regard to both activity and mRNA content during developmental and pathological conditions will provide new insight into the influence of nutrient availability on intrauterine growth.

CHAPTER 4

DEVELOPMENTAL REGULATION OF PLACENTAL AND FETAL LIVER ANIONIC AMINO ACID TRANSPORT AND THE CHARACTERIZATION OF A RAT GLUTAMATE TRANSPORTER.

Introduction

Although the anionic amino acids, aspartate and glutamate, are considered non-essential in that both can be synthesized from metabolic precursors, the varied range of function of these amino acids ranks them among the most important. In the brain, aspartate and glutamate can function as excitatory amino acid neurotransmitters found in many central nervous system pathways (Kanai et al., 1994). However, both can be neurotoxic as well, such that the proper function of the transporters responsible for the clearance of these amino acids from the synaptic cleft is required to prevent neuronal damage. These amino acids also serve in nitrogen shuttle pathways between different organs in the body (Souba, 1987), as well as from mother to developing fetus (Vaughn et al., 1995). In spite of the importance of these amino acids and the recent cloning of several anionic amino acid transporters, the transport of these nutrients, especially with respect to development, has not been well defined.

Placental Anionic Amino Acid Transport

Most amino acids are concentrated in the fetus by the extraction of these nutrients from the maternal circulation via Na^+-dependent transporters on the apical membrane of the syncytiotrophoblast and the subsequent efflux through facilitated transporters on the basal membrane. Glutamate and aspartate represent two important exceptions to this general rule. It has been well documented in sheep and humans that there is no net transfer of anionic amino acids from maternal to fetal circulation (Eaton and Yudilevich, 1981; Yudelivich and Sweiry, 1985; Smith et al., 1993). However, there is considerable uptake of these same amino acids from the fetal circulation. Perfusion studies by Battaglia and coworkers in sheep identified a significant component of Na^+-dependent glutamate uptake in the placenta that was restricted to the fetal-facing basal membrane (Marconi et al., 1989). In fact, the trophoblast glutamate concentration is nearly 100-fold greater than either the maternal or fetal circulations (Phillips et al., 1978).

Concomitant with glutamate and aspartate uptake into the placenta, the metabolically related amino acids glutamine and asparagine demonstrate significant efflux into the fetal circulation (Smith et al., 1993). As these data appear to support a nitrogen cycle between the placenta and fetal circulation, Vaughn and coworkers (1995) examined the transport and conversion of maternally-infused glutamine to glutamate in the sheep. A net flux of glutamine from the placenta to the fetal circulation was observed with a parallel net flux of glutamate from the fetus to the placenta. These data supported previous findings by this laboratory (Moores et al., 1994) and other investigators (Heittmann and Bergman, 1981). It was also noted that over 50% of the fetal circulating glutamine was extracted by the fetal liver with the resulting efflux of glutamate (Vaughn et al., 1995). This glutamate was then extracted by the placenta and surprisingly not used for the synthesis of glutamine, but rather oxidized in the placenta (Moores et al., 1994).

Limited studies with respect to placental anionic amino acid transport separated from metabolism have been reported. As was the case for cationic amino acid transport in the placenta (Chapter 3), the data presented by Kudo and Boyd (1990) in human apical and basal membrane vesicles is difficult to interpret due to the excessively high inhibitor concentrations. More detailed and useful analysis was provided in two reports by the laboratory of Carl Smith (Moe and Smith, 1989; Hoeltzli et al., 1990). Isolation of membrane vesicles from human term placentas revealed, on the apical membrane, a single Na^+-dependent system for anionic amino acids (Moe and Smith, 1989). This activity was dependent on K^+ efflux. The activity was also electrogenic, in that, as the net negative charge increased intravesicularly, the anionic amino acid uptake increased. This is due to the movement of a single positive charge (Na^+) into the vesicle with every molecule of substrate transported (Hediger et al., 1993). The inhibition profile suggested that the placental transport activity resembled the previously kinetically defined System X^-_{AG} (Gazzola et al., 1981). The identification of glutamate transport activity on the apical membrane suggested clearance of this substrate from the maternal circulation. However, as was discussed above, no net flux is seen to the fetus. A similar Na^+-dependent anionic amino acid transport activity was also described on the basal membrane of the human trophoblast consistent with the role of glutamate extraction from the fetal circulation (Hoeltzli et al., 1990). No reports have been published on the description of anionic amino acid transport in rat placenta, and no reports document the development of this transport throughout gestation.

Liver Anionic Amino Acid Transport

The fetal liver in sheep and humans is seen as a net exporter of glutamate under standard physiological conditions (Marconi et al., 1989; Heittman and Bergman, 1983), and as such, there must be facilitated exchange mechanisms in place for the efflux of these amino acids. In early experiments with liver slices (Hems et al., 1968) and perfusion studies (Ross et al., 1967), no net flux of glutamate was observed. However, in isolated hepatocytes, Gebhardt and Mecke (1983) identified a large Na^+-dependent component of glutamate uptake. The reason for this discrepancy becomes clear in that as is discussed below, the Na^+-dependent glutamate activity of the adult liver is limited to the canilicular membrane domain of a small population of hepatocytes which were not exposed in the two studies discussed above.

In the liver, a zonal distribution of hepatocytes with unique function is seen and referred to as hepatocyte heterogeneity. Glutamine synthetase activity is found only in a small population (about 7% of total) of perivenous hepatocytes surrounding the hepatic vein and not in the majority of the periportal hepatocytes (Haüssinger, 1989a). These cells also represent more than 70% of the Na^+-dependent glutamate uptake seen in the liver and have at least 20-fold increased activity for these substrates compared to perivenous hepatocytes (Haüssinger et al., 1989b). It also appears that most of the glutamate in these cells is directed toward glutamine synthesis in that in the perivenous hepatocytes the urea cycle is not active (Haüssinger, 1989a), and perivenous hepatocytes have a limited capacity for gluconeogenesis (Jungermann and Katz, 1982). The periportal cells are not without glutamate uptake, however, most of the uptake in these cells is Na^+-independent (Lindros et al., 1985).

Interestingly, perivenous hepatocytes are not only the major site of Na^+-dependent uptake in the liver but also the major site of glutamate release, as well (Haüssinger et al., 1989a). The data presented by Haüssinger and coworkers (1989b) suggest the presence of a glutamate/glutamate exchange mechanism in perivenous hepatocytes based upon several observations. In perfusion studies, the rates of glutamate influx and efflux were similar when the influent perfusate contained physiological concentrations of glutamate (0.1 mM), and in the same system, unlabelled glutamate stimulated the efflux of pre-loaded [^{14}C]-glutamate. These findings may be related to the Na^+-independent system x_c^- of fibroblasts that mediates a glutamate/cysteine exchange system.

To extend the studies described above, Stoll and coworkers (1991) again utilized liver perfusion but transport in this case was detected by autoradiography. The results confirm the localization of Na^+-dependent glutamate uptake to the small population of perivenous hepatocytes.

In isolated membrane vesicles, Na^+-dependent glutamate transport was limited to the bile canalicular membrane domain suggesting a role for this transporter in the uptake of glutamate from the bile (Sipps et al., 1982; Low et al, 1992). In fact, the levels of glutamate in the bile are well in excess of the proposed K_m of this transporter and most likely keep it saturated.

The reason for the glutamate/glutamate exchange system in perivenous hepatocytes is unclear. However, it is clear that Na^+-dependent transport of glutamate seems to follow the hepatocyte population expressing glutamine synthetase. Fetal hepatocytes do not have a defined zonal distribution of glutamate utilizing enzymes. In fact, glutamine synthetase is expressed in nearly the entire hepatocyte population of the fetal liver (J. Neu, unpublished observations). One might then expect that Na^+-dependent glutamate transport would be more widespread in the fetal liver, and then exhibit some kind of co-regulation limiting its expression to the perivenous hepatocyte population throughout the development.

This section of my thesis aims to developmentally define the anionic amino acid transporter complement of both the fetal liver and the placenta in light of the roles of membrane transport in the proposed glutamine-glutamate cycle of Battaglia (1992). As discussed in Chapter 1, Na^+-dependent glutamate transport has been ascribed to three separate cDNAs (Storck et al., 1992; Pines et al., 1992; Kanai and Hediger, 1992). Quite recently, a fourth cDNA has been isolated (Fairman et al., 1995). For the first time, this study will attempt to correlate the regulation of transport activity to the particular cDNAs responsible for the activity.

Methods

Isolation of a Na^+-dependent glutamate transporter from rat brain. In collaboration with Bob Fremeau at Duke University, a full-length cDNA was isolated from a rat brain hippocampal library by degenerate PCR followed by cDNA library screening (Velaz-Faircloth et al., in press). Poly A^+ mRNA isolated from rat hippocampus was converted to singlestrand cDNA by reverse transcription by a protocol provided by the vendor (GibcoBRL). Degenerate oligonucleotide primers (5'- CTG ATG AGG ATG CTG AA -3', 5'- TGT GC(A/T/G) AT(A/G) AA(C/A/G) A(C/T)(T/G/C) GC(T/G) GC -3') corresponding to conserved amino acid sequences in previously cloned glutamate transporters (Arriza et al., 1993) were used for PCR (30 cycles, 94°C 1 min, 45°C 2 min, 72°C 3 min). This partial cDNA was sequenced to confirm identity to the previously cloned glutamate transporters (Kanai and Hediger, 1991; Arriza et al., 1993). The partial cDNA was then used to screen a rat brain hippocampal library in λZAP II (Stratagene). Positive plaques were picked and converted to plasmid (pBsSK) by in vivo excision as described by the vendor (Stratagene). The cDNA has been designated rat EAAC1 to confirm its relationship to the rabbit clone (Kanai and Hediger, 1991)

Expression of glutamate transport in HeLa cells. Transient transfection of HeLa cells was carried out as previously described (Fremeau et al., 1992; Velaz-Faircloth et al., in press). HeLa cells were plated at 150,000 cells per well on Costar 24-well cluster trays and infected with recombinant vaccinia virus (15 PFU/cell) expressing T7 RNA polymerase (vTF7-3, Fuerst et al, 1986). The cells were infected for 45 min at 37°C. The virus was then removed and replaced with serum-reduced minimal essential medium (OptiMEM) containing 12 μl lipofectamine / ml, 4 μg EAAC1 cDNA in pBsSK / ml and incubated for 8 hrs before assaying transport activity of 50 μM [^{3}H]-L-glutamate or L-aspartate as described in Chapter 2. Stable, episomal-expression of EAAC1 was conducted as described in Chapter 6 and transport measured in a similar fashion.

Bacterial expression of EAAC1-maltose binding fusion-protein. The C-terminal 120 amino acids of EAAC1 were cloned in-frame into the pMALc2 prokaryotic expression vector (New England Biolabs, Beverly, MA) by standard molecular biological methods (Sambrook et al., 1989). A single colony of E. coli (JM109), transformed with pMALc2-EAAC1, was used to inoculate a 15 ml overnight culture of LB broth supplemented with 100 μg/ml ampicillin (amp) for expression and purification of the EAAC1-maltose binding protein fusion (MBP-EAAC1)(Current Protocols, 16.6). A 10 ml aliquot of the overnight culture was added to 1000 ml LB broth (100 μg/ml amp) and incubated at 37°C with shaking to $OD_{600} = 0.5$. IPTG was added to a final concentration of 3 mM and the culture incubated at 37°C for an additional 2 hrs to induce the expression of MBP-EAAC1. The cells were then pelleted by centrifugation and resuspended in 50 ml column buffer (20 mM Tris-Cl, 0.2 M NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, pH 7.4). Cell membranes were then disrupted by probe sonication at 50% duty cycle for 1 min. Release of cellular protein was monitored by assaying an aliquot of cell supernatant for total protein by the Lowry method (Lowry, 1951). The remaining steps were carried out at 4°C unless otherwise indicated. Cellular debris was pelleted by centrifugation and the supernatant diluted with 5 volumes of column buffer before application to an 8 ml pre-equilibrated amylose column (New England Biolabs). The fusion protein was released from the column by elution with 2 mM maltose. The MBP-EAAC1 fusion protein was then concentrated and combined with Freunds Adjuvant for polyclonal antibody production by immunization of rabbits at CoCalico Biological (Reamstown, PA).

Immunofluorescent labelling of cells with EAAC1 antibody. Cells episomally expressing EAAC1 (293c18-EAAC1) were grown on poly-L-lysine coated coverslips for immunofluorescence as described previously (Woodard et al., 1993). In brief, adherent cells were fixed in 4% paraformaldehyde, and non-specific protein binding blocked with 10% normal goat serum in PBS. Primary antibody, diluted in 10% normal goat serum in PBS supplemented with 0.1% Triton X-100, was incubated with the adherent cells for 1 hr at 37°C in a humidified chamber. After repeated washings in PBS, secondary antibody (goat α-rabbit FITC conjugate) was applied in a similar fashion. Fluorescent cells were visualized by indirect-immunofluorescence, and cell morphology was observed by phase contrast microscopy of the labelled cells.

Glutamate transporter isoform rtPCR. One microgram of poly A^+ mRNA was converted to cDNA by reverse transcription per the protocol supplied by the vendor (GibcoBRL, Gaithersburg, MD). PCR primers were chosen based on published sequences of the glutamate transporter clones as

follows: rat **GLAST1** (5′- TTG GAT TTG CCC TCC GAC CG -3′, 5′- GGT GCA TAC CAC ATT ATC ACC GC -3′); rat **GLT1** (5′- ATG GCA TCA ACC GAG GGT GC -3′, 5′- CGT GTC GTC ATA AAC GGA CTG CG -3′); rat **EAAC1** (5′- ATT CCT CAC AAA CGC CGG TGC -3′, 5′- GCT CCT TAA CGC TGG AGT TGG C -3′); human **EAAT4** (5′- TGC GCC CAT ATC AGC TCA CCT ACC -3′, 5′- TGC CCA GCC TCA TAA TAG CC -3′). Primers were checked for specificity against the other glutamate transporter cDNAs by computer analysis (PCGene, Intelligenetics). Brain cDNA was used as a positive control to optimize the PCR conditions. Thermal cycling using Taq polymerase (GibcoBRL) included 25 cycles of 94°C for 1 min, 60°C for 1 min 30 sec, 72°C for 2 min. All reactions were brought to 4 mM $MgCl_2$, and the EAAC1 reactions supplemented with 10% DMSO. Specificity of the PCR products was suggested by a single species of predicted size for each primer set and confirmed by unique restriction endonuclease site analysis.

Results

Sequence analysis of rat EAAC1 cDNA. The nucleotide sequence of the EAAC1 clone identified an open reading frame coding for 523 amino acids and predicted a core molecular mass of 57 kDa (Figure 4-1) The deduced amino acid sequence was highly conserved when compared to the homologous EAAC1 transporters previously isolated from rabbit intestine (Kanai and Hediger, 1991) and human brain (Kanai et al., 1994). Similar tertiary structure was also predicted with 12

Figure 4-1. Deduced amino acid sequence of rat EAAC1 cDNA. Alignment of the amino acid sequence or rat, rabbit (Kanai and Hediger, 1992), and human (Kanai et al., 1994) EAAC1 glutamate transorters. Shown are the deduced amino acid sequences from the reported cDNA sequences. Amino acid sequence identity is indicated by an asterisk. Alignment was performed by the Clustal program of the PCGene sequence analysis package (Intelligenetics Inc.) Putative N-linked glycosylation sites are marked by arrowheads and putative hydrophobic streches (membrane spanning regions) are indicated by black bars. The rat EAAC1 nucleotide sequence is deposited with GenBank under the accession number U21107.

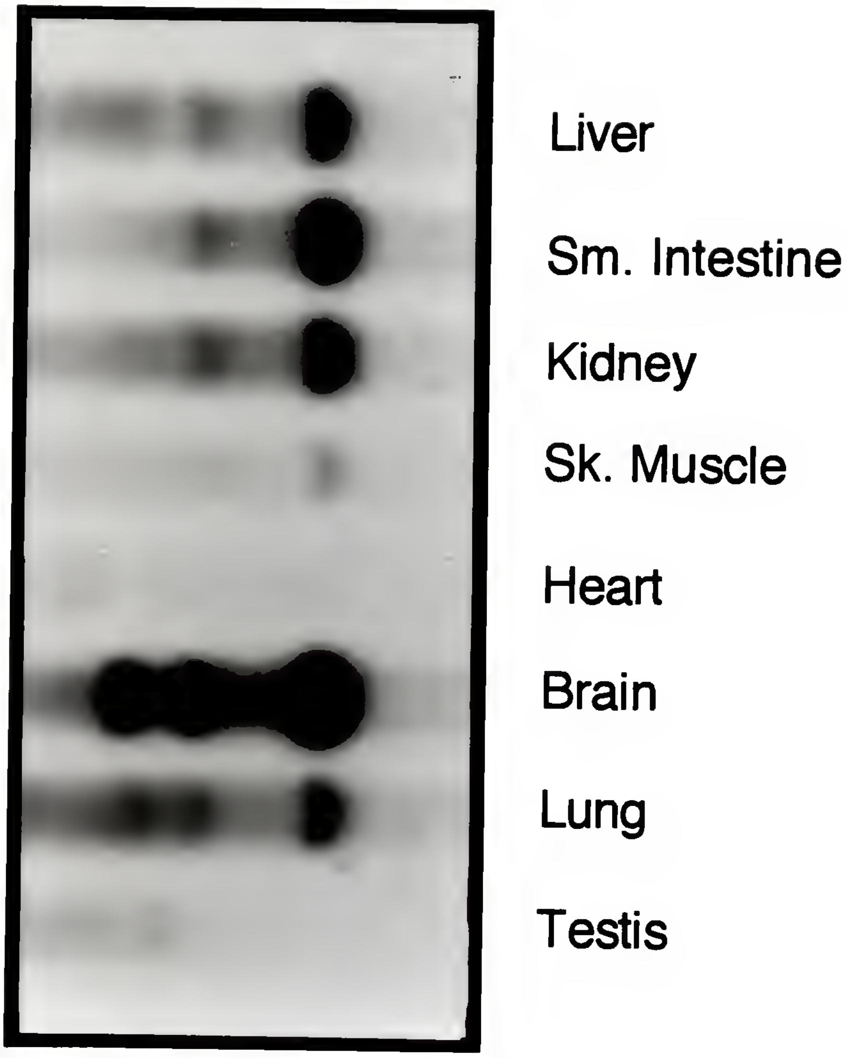
```
                                       1
Rat         MGKPTSSGCDWRRFLRNHWLLLSTVAAVVLGIVVGVLVRGHSELSNLDKF
Rabbit      ****ARK***SK***K*N*******V*******I*****EY*N**T****
Human       ****ARK**E*K***K*N*V************TT*****E**N**T****
                                2                 ↓
Rat         YFAFPGEILMRMLKLVILPLIISSMITGVAALDSNVSGKIGLRAVVYYFS
Rabbit      *********************V***********************L***C
Human       ***************I*****************************L***C
            3                          ↓
Rat         TTVIAVILGIVLVVSIKPGVTQKVNEINRTGKTPEVSTVDAMLDLIRNMF
Rabbit      **I*********************D**D***S******************
Human       **L*********************G**A***S******************
                                       ↓                ↓
Rat         PENLVQACFQQYKTKREEVKPASDPGGNQTEVSVTTAMTTM-SENKTKEY
Rabbit      **************T****TASD*T*K*G**E***AV***AV***R****
Human       *******************N*****EM*M**E*F*AV***AI*K******
                              4
Rat         KIVGLYSDGINVLGLIIFCLVFGLVIGKMGEKGQILVDFFNALSDATMKI
Rabbit      RV**************V*********************************
Human       ****M***********V*********************************
                        5                                    6
Rat         VQIIMCYMPIGILFLIAGKIIEVEDWEIFRKLGLYMATVLSGLAIHSLVV
Rabbit      *********L*************************************I*I
Human       *********H*************************************I*I
                                     7
Rat         LPLIYFIVVRKNPFRFALGMAQALLTALMISSSSATLPVTFRCAEEKNHV
Rabbit      *****************M**T***************************R*
Human       *****************M****************************N*Q*
                                8
Rat         DKRITRFVLPVGATINMDGTALYEAVAAVFIAQLNGMDLSIGQIITISIT
Rabbit      ***********************************D************V*
Human       ***********************************DL**G********V*
            9                            10
Rat         ATAASIGAAGVPQAGLVTMVIVLSPVGLPAEDVTLIIAVDWLLDRFRTMV
Rabbit      ************************A*************************
Human       **S*********************************************V*

Rat         NVLGDAFGTGIVEKLSKKELEQVDVSSEVNIVNPFALEPTILDNEDSDTK
Rabbit      **********************M***************SAT*********
Human       **************************************S***********

Rat         KSYVNGGFSVDKSDTISFTQTSQF  -523
Rabbit      ***I****A***************  -524
Human       ********A***************  -524

Identity -vs- Rabbit:      90.1%
Identity -vs- Human:       91.0%
```

Figure 4-2. Tissue distribution of rat EAAC1 mRNA. Poly A^+ mRNA (3 μg per lane) was separated by 1% agarose gel electrophoresis and transferred to nylon membrane. The filter was hybridized with ^{32}P-labelled EAAC1 cDNA probe and washed at 65°C (375 mM NaCl, 40 mM Na_2HPO_4, pH 7.2, 1 mM EDTA, 1% SDS). The filter was stripped and hybridized with ß-actin cDNA probe to confirm mRNA quality and loading in each lane.


Liver
Sm. Intestine
Kidney
Sk. Muscle
Heart
Brain
Lung
Testis

hydrophobic stretches of which at least the C-terminal six are strongly suggested to span the membrane by computer prediction (PCGene, Intelligenetics). The sequence also predicts six potential protein kinase C phosphorylation sites and four potential N-linked glycosylation sites (Asn-X-Ser\Thr).

Tissue distribution of rat EAAC1. Figure 4-2 represents a multiple tissue Northern blot hybridized with a ^{32}P-labelled EAAC1 cDNA probe under high stringency conditions. Three mRNA species were detected: a prominent band at 4.0 kb and two bands at 2.8 and 2.2 kb of lower intensity. The greatest hybridization is seen in brain consistent with previously published reports (Kanai and Hediger, 1992; Kanai et al., 1994). Hybridization was also seen in small intestine, kidney, lung, and weak hybrization in liver.

Expression of rat EAAC1. Anionic amino acid transport activity mediated by the rat EAAC1 cDNA was examined in cells both transiently and stably transfected. Na^+-dependent uptake of 50 μM [^{3}H]-aspartate or -glutamate was significantly higher than vector-only transfected controls and represented a 20-fold and 40-fold increase over controls in transiently and stably transfected cells, respectively (Table 4-1). The EAAC1-mediated uptake was completely inhibited by L-glutamate (96%) and D- and L-aspartate (88% and 92%) consistent with previously published reports for EAAC1 (Kanai et al., 1994).

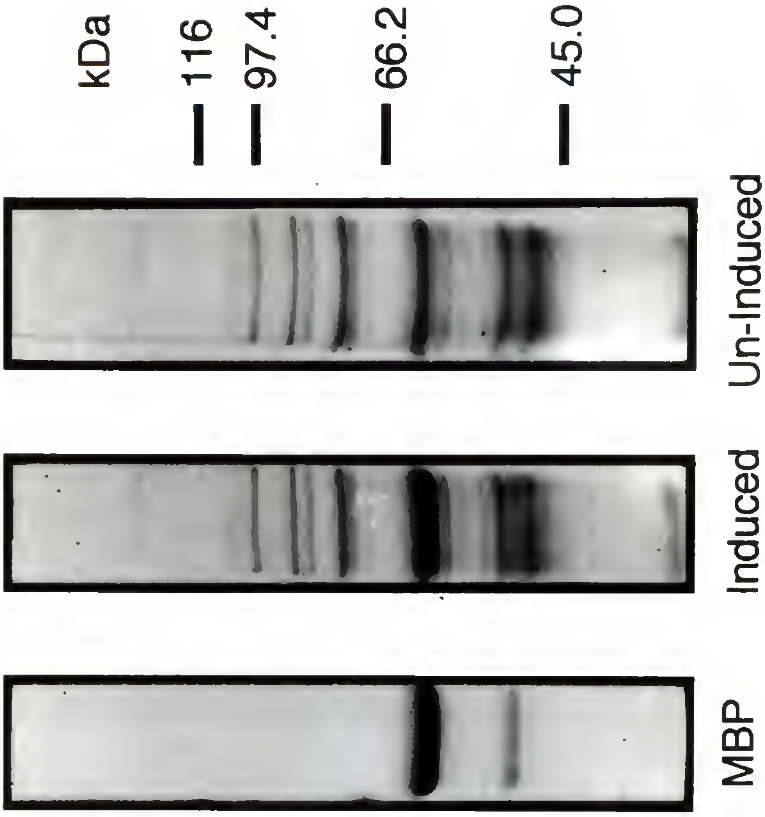
Generation of polyclonal antisera to rat EAAC1. In order to generate a high titer antisera for detection of EAAC1

Table 4-1. Transient and stable expression of rat EAAC1.

	Transient (pCDNA)	Stable (pDR2)
Control	50.2 ± 8.2	61.1 ± 4.6
EAAC1	1105.9 ± 76.5	2396.1 ± 209
EAAC1 + L-glutamate		95.8 ± 7.6
EAAC1 + L-aspartate		287.6 ± 32.5
EAAC1 + D-aspartate		191.7 ± 9.7

The values are means (n = 3) ± SEM in pmol·mg^{-1} protein·min^{-1}. Uptake of 50 μM [^{3}H]-aspartate was assayed for 1 min at 37°C as described in text. Inhibitors were included at 5 mM.

Figure 4-3. Synthesis and purification of MBP-EAAC1 fusion protein. Bacteria were transformed with the pMAL-C2 MBP fusion protein vector containing the nucleotide sequence corresponding to the C-terminal 120 amino acids from rat EAAC1. "Un-induced" and "Induced" represent cell extracts before and after incubation with 3 mM IPTG, respectively. "MBP" represents the pooled peak fraction eluate from an amylose affinity column after application of 2 mM maltose. The lower (≈50 kDa) band in the "MBP" lane has been identified upon further experimentation as either a MBP-only product resulting from either degredation or incomplete synthesis of the fusion protein.


kDa
116
97.4
66.2
45.0
Un-Induced
Induced
MBP

protein, nucleotides corresponding to the C-terminal 120 amino acids of the deduced sequence were expressed as a fusion protein in E. coli. After induction of fusion protein expression by IPTG, the bacteria produced the MBP-EAAC1 fusion protein at high levels (Figure 4-3). Of particular concern with regard to the expression of this and other membrane-bound proteins is the insolubility due to the extensive hydrophobic character of the protein. Even though there were stretches of hydrophobic residues in the C-terminal EAAC1 sequence, the 42 kDa MBP was able to keep the fusion protein soluble rather than as inclusion bodies in the bacteria. The resultant polyclonal antiserum detected a single immunoreactive polypeptide in 293c18 cells expressing EAAC1 consistent with the increase in transport activity observed after transfection (Figure 4-4). The specificity of the detected species was confirmed by the specific competition of immunoreactivity by the MBP-EAAC1 fusion protein, while no competition was seen with MBP alone (Figure 4-5).

Developmental regulation of placental glutamate uptake. Chapter 3 described the developmental regulation of cationic amino acid uptake in both apical and basal membrane vesicles from placentas at 14 to 20 days gestation. It was seen that specific transport systems for arginine increased with increasing gestational age to meet the demands of the developing fetus which will double its weight during this final period of gestation. With the increased demand for

Figure 4-4. Stable expression of rat EAAC1 in human 293c18 cells. (Left panel) The Na^+-dependent uptake of 50 μM [^{3}H]-aspartate was assayed at 37°C for 1 min. Values represent the mean ± SD for quadruplicate determinations from cells transfected with vector only (pDR2) or rat EAAC1 (pDR2/EAAC1). (Middle panel) Plasma membranes were subjected to 7.5% SDS-PAGE and immunoblotted with polyclonal antisera generated to the MBP/EAAC1 fusion protein. (Right panel) Northern analysis of total RNA (20 μg) probed with [^{32}P]-labelled EAAC1 cDNA.

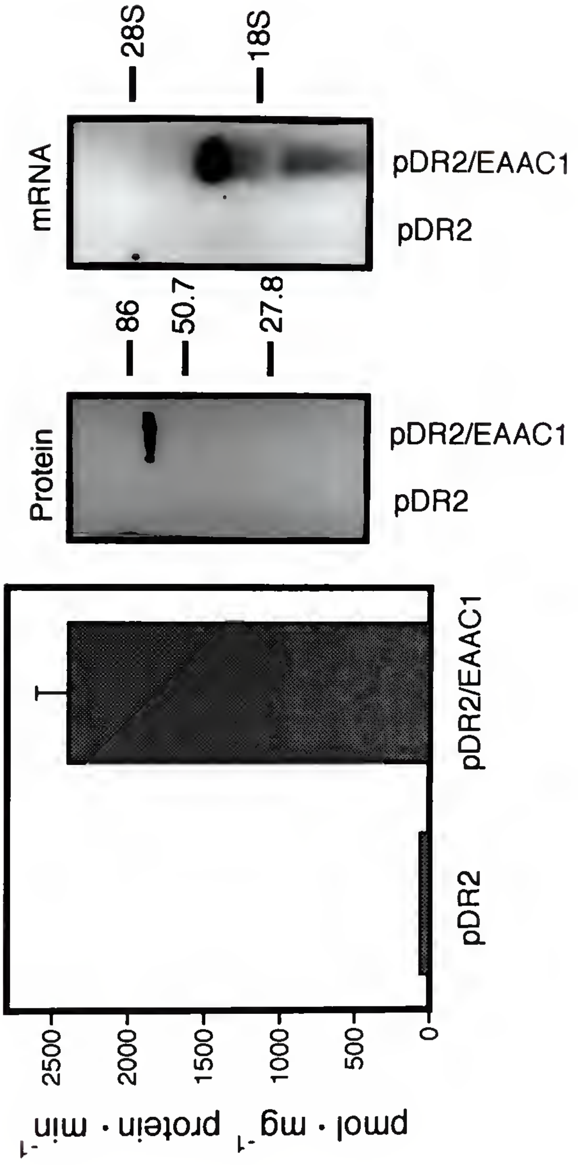
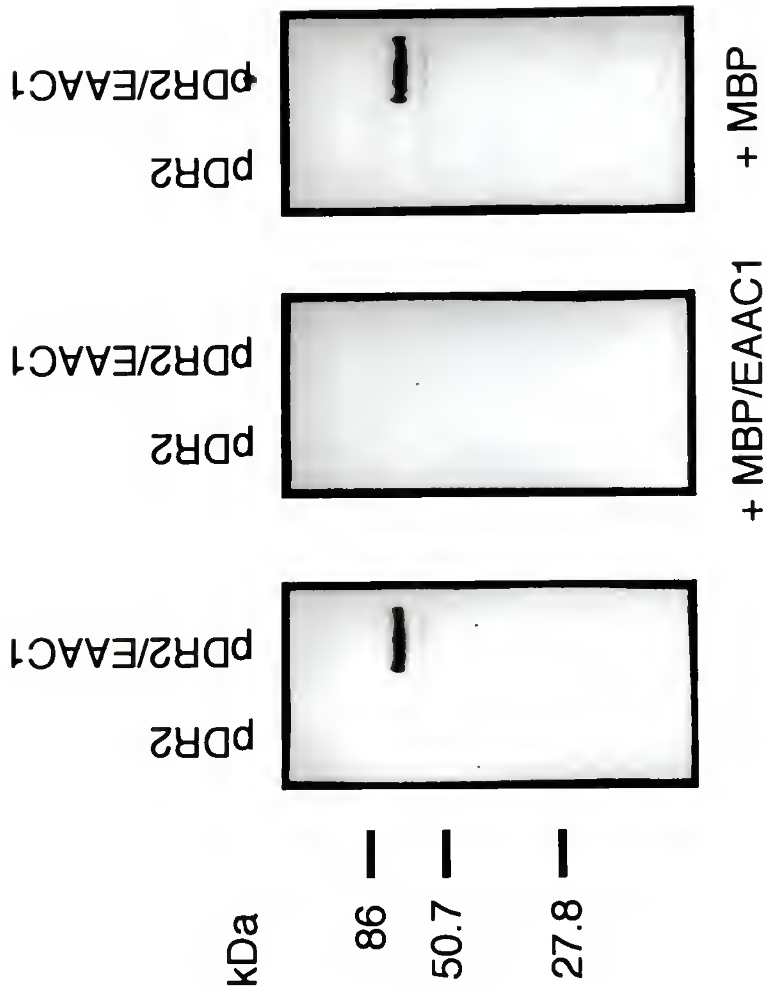

mRNA
28S
18S
pDR2/EAAC1
pDR2
Protein
86
50.7
27.8
pDR2/EAAC1
pDR2
pmol · mg⁻¹ protein · min⁻¹
2500
2000
1500
1000
500
0
pDR2
pDR2/EAAC1

Figure 4-5. Immunoblot analysis of over-expressed rat EAAC1. Human 293c18 cells were transfected with vector only (pDR2) or with the EAAC1 cDNA insert (pDR2/EAAC1). Plasma membranes were prepared by differential centrifugation and subjected to 7.5% SDS-PAGE. Electrotransferred membranes were probed with EAAC1 antiserum (1:500 in 5% CNFDM/TBS/0.1% Tween20). Immuno-reactivity was visualized by chemiluminescence. A single immunoreactive species is observed at ≈80 kDa. Competition the fusion protein (MBP/EAAC1) but not the MBP alone resulted in the loss of immunoreactivity suggesting specificity.


kDa
86
50.7
27.8
pDR2
pDR2/EAAC1
pDR2
pDR2/EAAC1
+ MBP/EAAC1
pDR2
pDR2/EAAC1
+ MBP

nutrients for both protein acretion and energy, one would expect the return of metabolic by products such as glutamate, the deamination product of glutamine, may be increased as well. As such, the uptake of 50 μM glutamate was examined in apical and basal vesicles similar to those described in Chapter 3. Na+-dependent glutamate transport was completely absent without intravesicular K^+. In all experiments, the vesicles were freeze-thaw pre-loaded with 100 mM KCl to provide K^+ efflux which has been demonstrated in human placenta to be absolutely required for anionic amino acid uptake (Moe and Smith, 1989; Hoeltzli et al., 1990). Glutamate uptake was Na^+-dependent and was higher in basal membrane compared to apical membrane vesicles at both 14 and 20 days gestation (Figure 4-6). Glutamate uptake also was increased at 60 sec in both apical and basal membrane vesicles from placentas at 20 days compared to 14 days gestation, no increase was noted at earlier time points.

Identification of placental glutamate transporter cDNAs. As at least four cDNAs have been isolated which mediate Na^+-dependent glutamate uptake, rtPCR analysis was used to identify which transporter clones are expressed in the placenta at both 14 and 20 days gestation. EAAC1 mRNA was the only glutamate transporter mRNA expressed at high levels in the placenta and was detected only from placentas of 20 days gestation (Figure 4-7). This result is consistent with the ubiquitous expression of this glutamate transporter cDNA

Figure 4-6. Time-course of [^{3}H]-glutamate uptake in basal or apical membrane vesicles from placentas of 14 (TOP) or 20 (BOTTOM) days gestation. Vesicles were preloaded with 100 mM KCl by freeze-thaw and incubation at 37°C for 30 min. Transport of 50 μM [^{3}H]-arginine was performed at 37°C in uptake buffer containing a final concentration of either 100 mM NaSCN (open circles) or 100 mM KSCN (closed circles). Values are reported as the means ± standard deviations for triplicate determinations. Where not shown, the standard deviation bars are contained within the symbol.

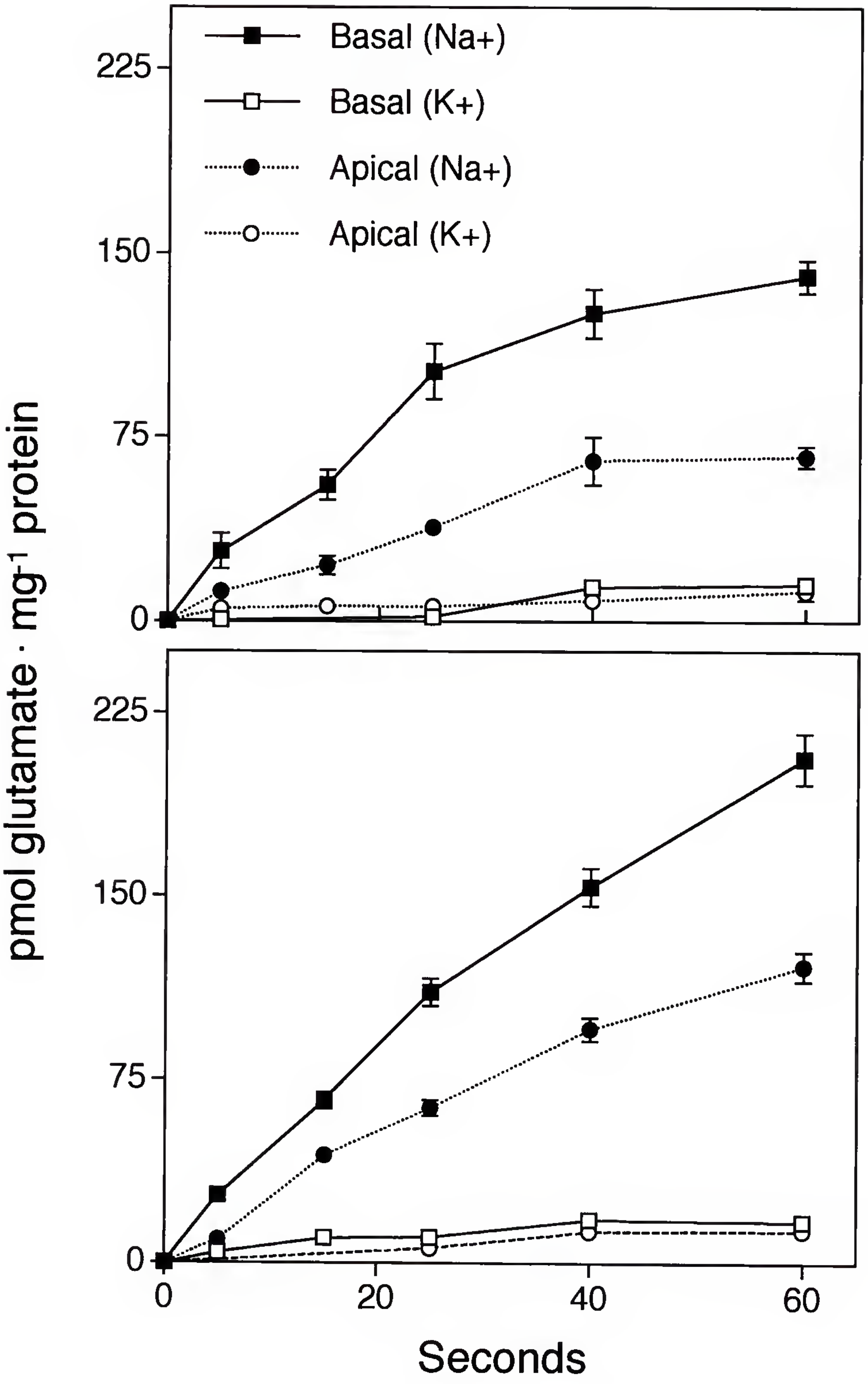
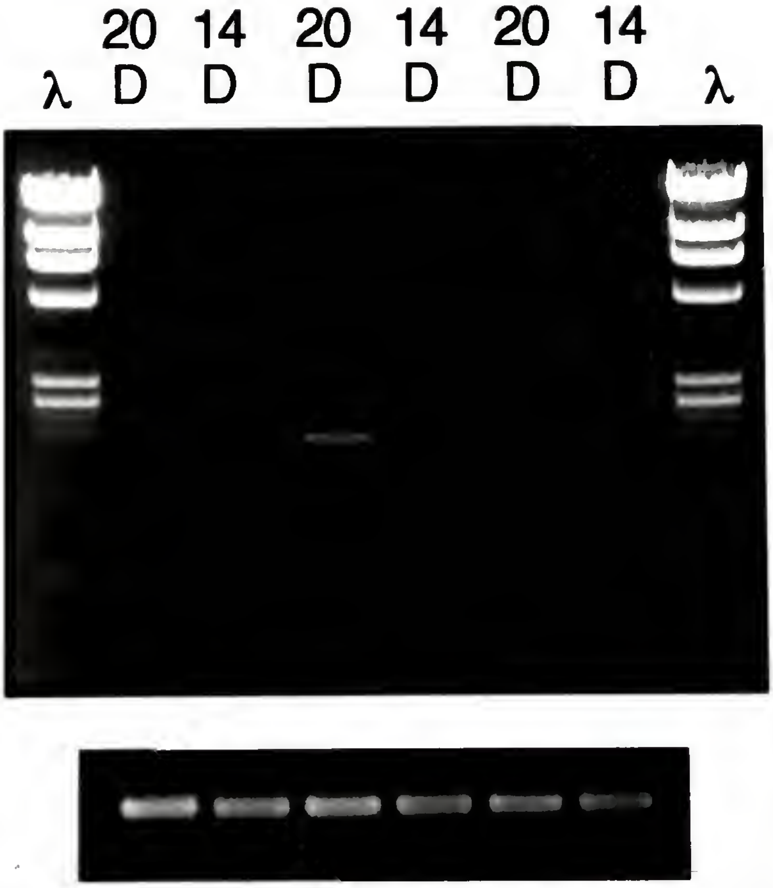

Basal (Na+)
Basal (K+)
Apical (Na+)
Apical (K+)
225
150
75
0
225
150
75
0
0
20
40
60
Seconds
pmol glutamate · mg^{-1} protein

Figure 4-7. Reverse transcriptase PCR detection of glutamate transporters in placentas at 14 (14D) and 20 (20D) days gestation. Pairs of 14D and 20D PCR products represent from left to right GLT1 primers, EAAC1 primers, and GLAST1 primers. One microgram of placental poly A^+ mRNA was converted to cDNA and subjected to PCR analysis as described in text with primers specific to each of the glutamate transporters. The 20 μl of the PCR reaction was subjected to 1.2% agarose gel electrophoresis and detected with ethidium bromide. The λ lane represents 1 μg of a Hind III digest of λ DNA.


λ
20 D
14 D
20 D
14 D
20 D
14 D
λ

(Kanai and Hediger, 1992). Low levels of GLAST1 mRNA were also observed.

Developmental regulation of EAAC1. Although the rtPCR used in the above assay was semi-quantitative, Northern blot analysis was used to examine the steady-state levels of EAAC1 mRNA and to confirm the lack of this transporter at 14 days gestation. Three mRNA species (4.0, 2.8, 2.2 kb) were detected in poly A^+ mRNA isolated from placentas of 20 days gestation (Figure 4-8) as was seen in the multiple tissue Northern blot described above (Figure 4-2). A very weakly-hybridizing signal could be detected in mRNA from placentas of 14 days gestation after over-exposure of the auto-radiographic film (15 days). These data confirm the rtPCR analysis of EAAC1 and suggest developmentally regulated expression of this transporter in the placenta. To examine EAAC1 protein levels, the polyclonal antisera generated above was used for immunoblot analysis of apical and basal membrane vesicles prepared from placentas of 14 and 20 days gestation. At 20 days gestation, both apical and basal membrane vesicles demonstrated an immunoreactive species consistent in size (82 kDa) with membrane vesicles prepared from EAAC1-expressing 293c18 cells (Figure 4-9). This immunoreactive band is also competable with the MBP-EAAC1 fusion protein suggesting specificity of detection. Another smaller immunoreactive species is seen in these samples as well. It most likely represents either a degradation product of EAAC1 or a protein with a shared epitope as the immunoreactivity is also

Figure 4-8. Northern analysis of EAAC1 mRNA from placentas of 14 (14D) or 20 (20D) days gestation. Poly A^+ placental RNA (3 μg per lane) was separated by 1% agarose gel electrophoresis and transferred to nylon membrane. The filter was hybridized with ^{32}P-labelled System y^+ cDNA probe and washed at 65°C. Three mRNA species are detected at 20 days gestation (4.0, 2.8, 2.2 kb). Overexposure for 15 days detects the same species at 14 days gestation. Values are representative of four independent experiments.

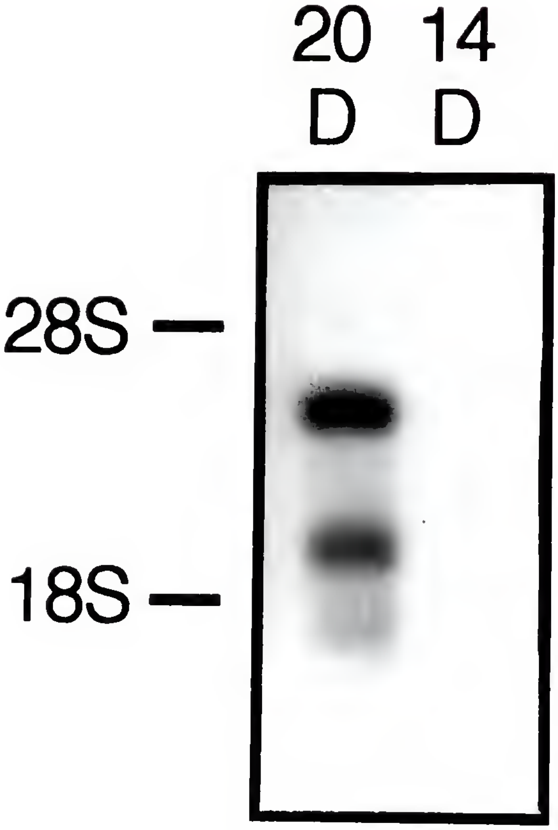
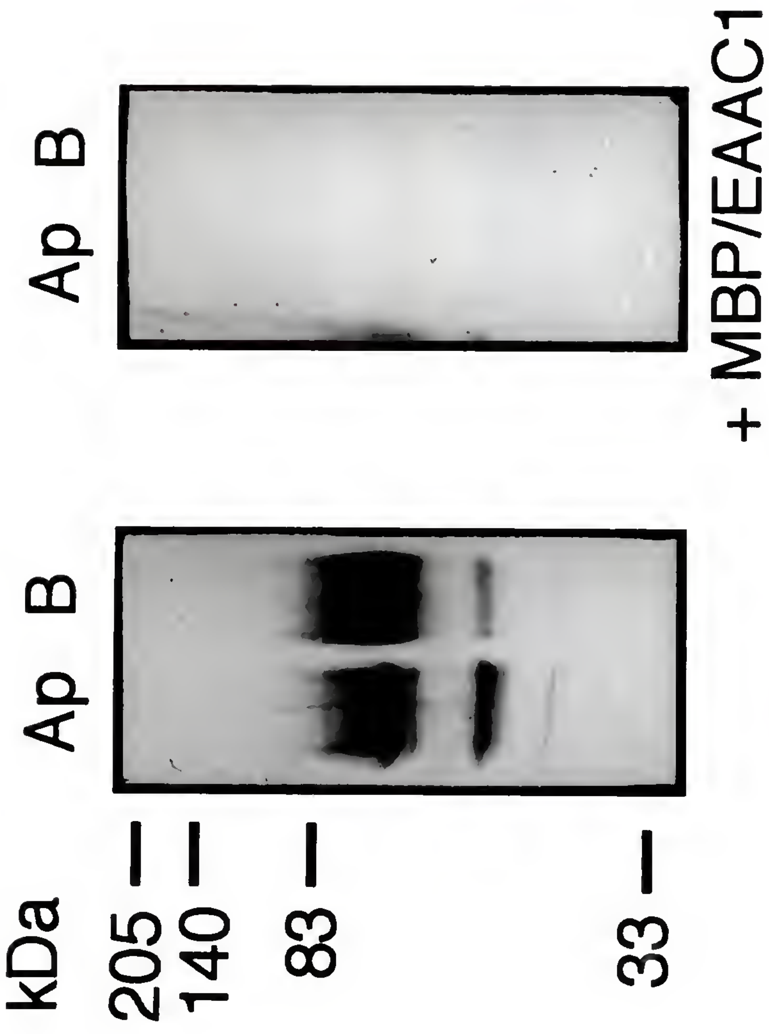

20
D
14
D
28S
18S

Figure 4-9. Immunoblot analysis of EAAC1 protein in apical (Ap) and basal (B) membrane vesicles from placentas of 20 days gestation. Isolated membrane vesicles (40 μg) were subjected to 7.5% SDS-PAGE. Proteins were electrotransferred to nitrocellulose membrane and incubated with EAAC1 antiserum. Immuno-reactivity was visualized by chemiluminescence. The major immunoreactive species is observed at ≈80 kDa corresponding to the species detected in over-expressing 293c18 cells (pDR2/EAAC1). Competition with the fusion protein (MBP/EAAC1) resulted in the loss of immunoreactivity suggesting specificity.


kDa
205
140
83
33
Ap
B
Ap
B
+ MBP/EAAC1

competed by the MPB-EAAC1 fusion protein. Consistent with the mRNA analysis, only weak immunoreactivity is seen in 14 day gestation vesicles (Figure 4-10). Collectively, these data suggest developmental regulation of the EAAC1 transporter protein in placenta from 14 to 20 days gestation.

Developmental regulation of fetal liver glutamate uptake. As the liver is the primary source of energy and protein acretion in the developing fetus (Gresham et al., 1972), glutamate uptake was examined in hepatocytes isolated from fetuses of increasing gestational age. As hepatocytes before 16 days gestation are considered more hematopoetic than parenchymal, analysis was limited to hepatocytes from fetuses of 18, 20 and 21 days gestation and 3 days after birth and adults. Significant Na^+-dependent uptake is observed in fetal hepatocytes with peak activity seen at 20 days gestation (Figure 4-11). Transporter expression then appears to switch from Na^+-dependent activity to Na^+-independent activity for the remainder of development, such that nearly all glutamate uptake in the adult hepatocytes is Na^+-independent. In an effort to examine if this regulation of Na^+-dependent anionic amino acid transport acitivity is due to decreased levels of the transporter mRNA and protein, rtPCR analysis, similar to that described above for placental RNA, was used to identify the transporter cDNAs responsible for the Na^+-dependent transport in fetal liver. Surprisingly, the only mRNA present was that of GLAST1 (Figure 4-12) formerly thought to be brain-specific (Storck et al., 1992).

Figure 4-10. Immunoblot analysis of EAAC1 from placentas of 14 and 20 days gestation. Apical (14Ap, 20Ap) and basal (14B, 20B) membrane vesicles (40 μg) were subjected to 7.5% SDS-PAGE. Proteins were electrotransferred to nitrocellulose membrane and incubated with either immune or preimmune serum. Immuno-reactivity was detected by chemiluminescence.

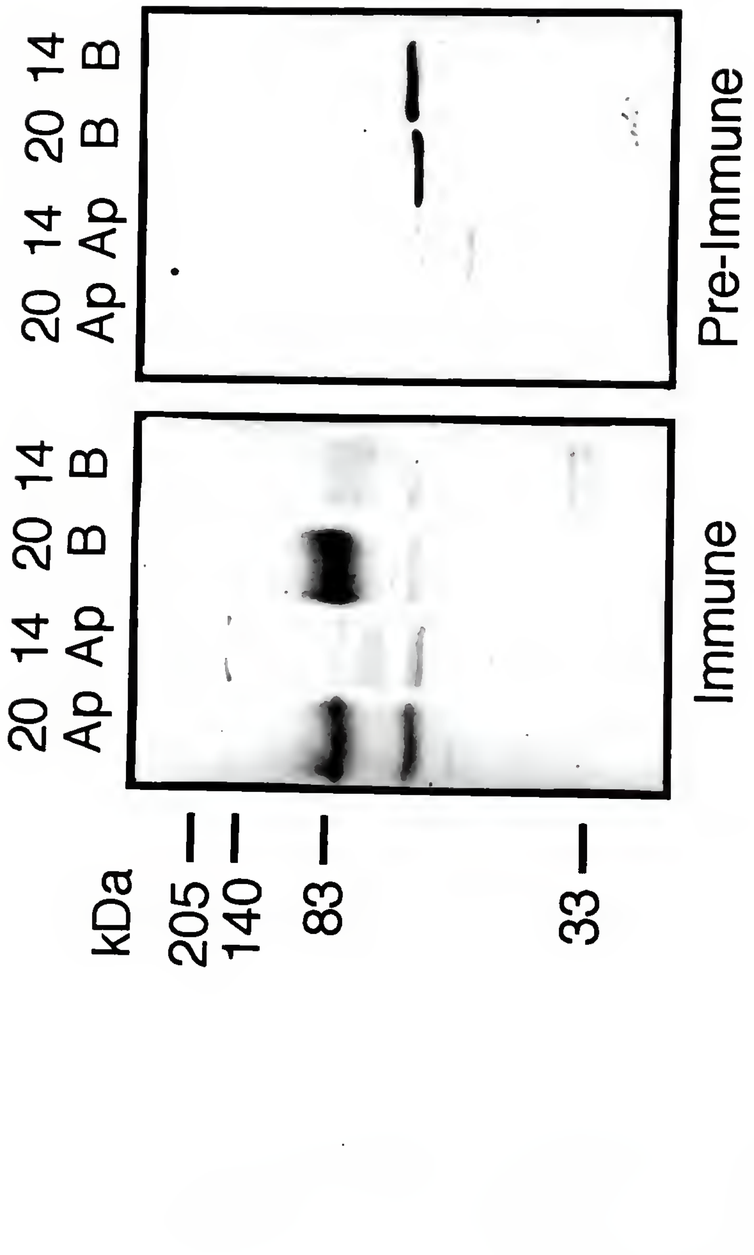

20 Ap
14 Ap
20 B
14 B
20 Ap
14 Ap
20 B
14 B
kDa
205
140
83
33
Immune
Pre-Immune

Figure 4-11. Uptake of glutamate in fetal, newborn, and adult hepatocytes. Transport of 50 μM [^{3}H]-glutamate was performed for 1 min at 37°C as described in text in Na^+KRP or CholKRP. Values represent mean ± SD of triplicate determinations from 3 independent experiments.

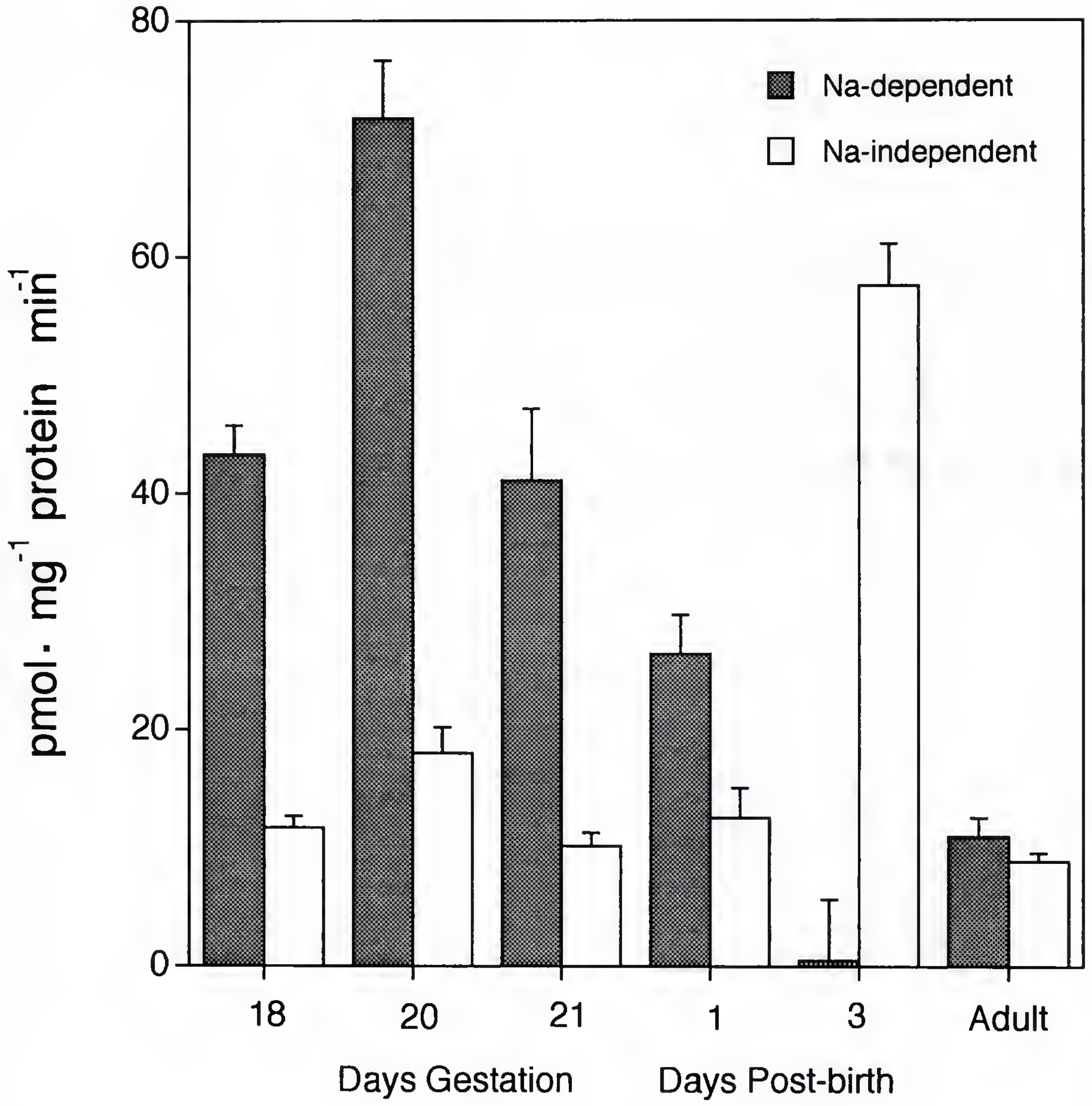

80
60
40
20
0
pmol · mg^{-1} protein min^{-1}
Na-dependent
Na-independent
18
20
21
1
3
Adult
Days Gestation
Days Post-birth

Figure 4-12. Reverse transcriptase PCR detection of glutamate transporters in fetal hepatocytes, 20 (20D) days gestation, and adult hepatocytes. One microgram of hepatocyte poly A^+ mRNA was converted to cDNA and subjected to PCR analysis as described in text with primers specific to the GLAST1 glutamate transporter. The 20 µl of the PCR reaction was subjected to 1.2% agarose gel electrophoresis and detected with ethidium bromide. The λ lane represents 1 µg of a Hind III digest of λ DNA.

20
D
λ

These results were confirmed with immunoblot and Northern blot analysis using EAAC1 antibody and cDNA, respectively. No EAAC1 protein or mRNA could be detected in the fetal or adult hepatocytes.

Discussion

Placental anionic amino acid uptake is unique in that there is no net concentration of these amino acids in the fetus (Eaton and Yudilevich, 1981; Yudelivich and Sweiry, 1985; Smith et al., 1993). Rather, the basal (fetal-facing) membrane of the trophoblast extracts glutamate and aspartate from the fetal circulation by Na^+-dependent transporters on this membrane face. Most of the previously described data have been obtained in the sheep model and in term human placentas. No reports exist on the transport of these substrates in the rat placenta.

My studies have demonstrated that glutamate uptake in the rat placenta is mediated by Na^+-dependent transporters present on both the apical and basal membranes of the syncytiotrophoblast. This transport is also dependent on K^+ efflux. These data are similar to these obtained in human vesicle preparations from apical and basal membranes (Hoeltzli et al., 1989; Moe and Smith, 1990), and further underscore the functional similarity between the human and rat placentas. Although the placenta extracts anionic amino acids from the fetal circulation, the role of the Na^+-dependent mechanism on the apical membrane is unclear. This may serve to maintain the high glutamate content of the trophoblast relative to both the maternal and fetal circulations (Phillips et al., 1978), and provide substrate for this rapidly oxidizing tissue. Some studies have also suggested that the high placental oxidation rates of glutamate also serve to provide co-factors (NADPH) necessary for hormone synthesis and release in the placenta.

No molecular evidence has been provided for anionic amino acid transport in the placenta. While the identification of EAAC1 mRNA and protein may not be surprising due to its ubiquitous expression, the detection of these components is completely novel in the field of placental amino acid transport and represents elevation of this field to the molecular level. The EAAC1 protein appears to be highly glycosylated in that the migration of the protein at 82 kDa is well above the core size predicted by amino acid sequence alone. Heterogenous glycosylation, as is the case for the facilitated glucose transporters (McMahon and Frost, 1995), is also suggested because the EAAC1 species migrates as a broad immunoreactive band. The identification of this protein on both the apical and basal membranes further serves to justify the assignment of Na^+-dependent glutamate transport to these two membrane vesicle populations.

As the metabolic demands of the fetus are the greatest near the end of the gestational period, one might expect that delivery of nutrients and the removal of metabolic by products would be highest during this period as well. This is what is observed with respect to glutamate transprot over gestation. Both the EAAC1 mRNA and protein are increased with increasing gestational age in the placenta. Also, transport of these amino acids is also increased in the fetal liver, peaking at 20 days gestation. Although the fetal liver is a net exporter of glutamate, careful examination of this tissue in the adult animal has clearly demonstrated a glutamate/ glutamate exchange in perivenous, glutamine synthetase containing hepatocytes (Haüssinger et al., 1989b). For net transport of glutamate into hepatocytes with a high intracellular glutamate concentration to occur, Na^+-dependent mechanisms would be employed. As only about 7% of all hepatocytes in the adult are perivenous in nature, the Na^+-dependent transport of anionic amino acids is not readily observed (Stoll et al., 1991). However, glutamine synthetase is expressed throught the fetal liver (J. Neu, unpublished observations), and by analogy, one might expect Na^+-dependent glutamate transport to be widely expressed as well. This is exactly the case that is seen in this study with a high level of Na^+-dependent anionic amino acid transport in fetal hepatocytes. The placement of this transport is further supported by the detection of GLAST1 mRNA in these hepatocytes as well.

Given that glutamate and aspartate are utilized in so many biochemical pathways, the ultimate fate of these substrates transported from the placenta is not yet known.

However, the unambiguous placement of transporter mRNA and protein may serve to at least suggest a role for these nutrients in various tissues.

CHAPTER 5
THE EFFECTS OF MATERNAL PROTEIN RESTRICTION ON PLACENTAL AMINO ACID TRANSPORT IN THE RAT

Introduction

In humans, intrauterine growth retardation (IUGR) affects millions of pregnancies, perhaps as many as 5% of births in the United States (Golde, 1989). The deleterious effects of IUGR are not limited to gestational development, but rather are seen throughout the life of the child and may present as increased risk for coronary artery disease, diabetes, and stroke (Barker, 1994). Many factors can contribute to IUGR in humans ranging from pathologic conditions to alcohol, cocaine, and tobacco abuse (Sastry et al., 1989). Perhaps the most logical factor is poor maternal nutrition. It is not unreasonable to assume that because the maternal circulation supplies nearly all nutrients needed for fetal development, a decrease in the available maternal amino acid pool would affect delivery to the fetus. However, the mechanism by which this decrease in the delivery of nutrients might occur has not been well described. The multifactorial origin of IUGR in the human makes the examination of the contribution of maternal nutrition difficult. In fact, nicotine, cocaine, and alcohol, factors often associated with poor nutrition during pregnancy, have all been suggested to affect placental nutrient transport (Karl and Fisher, 1994). It is for this reason we have chosen to investigate the effects of maternal malnutrition in a well characterized animal model.

Given that the vast majority of nutrients required by the fetus for normal growth and development are provided by the maternal circulation, it is not surprising that alteration of maternal nutrient pools could have possible deleterious effects on the fetus. Many investigators have documented that protein and protein-calorie malnutrition result in smaller fetuses with poor developmental outcomes (Naeye et al., 1973; Rosso, 1977). In fact, the effects of maternal malnutrition can be seen in adult offspring of these pregnancies. It appears that abnormal maternal nutrient status can "program" abnormal expression of several biochemical pathways in adult animals developing from small for gestational age pregnancies (Barker, 1994). However, the factors leading to growth retardation in the fetuses of malnourished mothers or the mechanism by which the inadequate fetal nutritional status is propagated into adulthood have yet to be described. Given that a depleted maternal nutrient pool could adversely affect the fetus, and as such, the examination of nutrient delivery in times of maternal malnutrition is important to elucidate some of these potential effects.

The placenta serves as the functional interface between maternal and fetal circulations and is responsible for nutrient delivery to the fetus by the combined actions of transport proteins on both the apical (maternal-facing) and basal (fetal-facing) membranes. Chapter 3 described the developmental regulation of cationic amino acid transport in the rat placenta, and during times of increased fetal nutritional need, the final third of gestation, the placenta up-regulates the production of cationic transport proteins to adequately supply the fetus. It follows that a diminuition of placental transport function could be detrimental to the developing fetus.

The role of placental amino acid transport in pregnancies complicated with maternal malnutrition has only been examined at the whole animal level. Initial observations into the effects of maternal nutrient status on transplacental nutrient flux was provided by Rosso (1975). Using a maternal bolus injection of nonmetabolizable, radiolabelled substrate, he demonstrated that rat dams deprived of protein and calories throughout gestation show a decreased delivery of 2-aminoisobutyric acid (AIB) to the fetus. AIB is a substrate of amino acid transport System A (Kilberg et al., 1993). Varma and Ramakrishnan (1991) examining a similar model of protein-calorie malnutrition have reported a similar decrease in System A activity. There was a significant decrease in the fetal to maternal distribution of AIB caused by both a decrease in clearance from the maternal circulation and an increase in the time required for efflux from the trophoblast. Contradictory data was provided by Ahokas and coworkers (1981), who saw no significant difference in the fetal delivery of AIB after maternal calorie deprivation. However, the model of nutrient depletion used by those investigators differed from both Rosso (1975) and Varma and Ramakrishnan (1991) and could be responsible for the apparent discrepancy.

Previous studies of protein and calorie deprivation in the rat model provide inferential evidence of decreased placental nutrient transport, but conflicting reports have been published. Also, all of the previous studies examined amino acid transport in models that were deficient in calories as well as protein. No data has been presented for amino acid transport in animals deprived of protein alone. The present study utilizes apical and basal enriched membrane vesicles to demonstrate that maternal protein deprivation causes a concurrent down-regulation of placental amino acid transport. The use of membrane vesicles removes potential secondary effects of diminished blood flow and metabolism, thereby isolating the contribution of transport proteins to this condition. Furthermore, the data represent the first reported analysis of steady-state mRNA levels in IUGR for selected transport proteins.

Results

Characterization of control (C) and low-protein (LP) groups. The LP fed dams gained significantly ($P < 0.005$) less weight during the gestational period than did the pair-fed C dams (Table 5-1). This reduction in weight gain can be attributed only to the decrease in protein content of the diet rather than calorie restriction as the two diets were isocaloric, and the mean daily food consumption of both the LP and C groups was the same (C 19.2 ± 1.3, LP 19.7 ± 1.6 g). Furthermore, there was no significant difference in litter size (Table 5-1) or in viability of the fetuses as assessed by visual inspection. There was an accompanying significant reduction ($P < 0.005$) in fetal and placental weight. These data, with respect to maternal and fetal weights, are in agreement with a previously described protein-calorie model of maternal malnutrition (Rosso, 1975). These data also demostrate that a decrease in dietary protein content alone is enough to elicit the development of growth retarded fetuses in this rat model.

Other investigators employing a similar diet formulation (Gomezangelats et al., 1995), argue against pair-feeding, suggesting that if the LP animals consumed an inadequate amount of food, all animals would be calorie-deprived. Several lines of evidence argue against calorie-deprivation in our study. First, the average daily food consumption (C 19.2 ± 1.3, LP 19.7 ± 1.6 g) is similar to the average daily food consumption of rats fed *ad libitum* over gestation (22.1 ± 3.2 g). Given the higher caloric composition of our diet (4.09 KCal/g) as compared to standard chow (3.34 KCal/g), these data argue against calorie-deprivation. Second, the values reported in this study for 50 μM [^{3}H]-MeAIB uptake via

Table 5-1. Maternal weight gain, fetal weight, placental weight, and litter size for control (C, 20% casein) and low-protein (LP, 5% casein) representing three independent experimental trials.

	MATERNAL WEIGHT GAIN(%)	FETAL WEIGHT	PLACENTAL WEIGHT	LITTER SIZE
C1	38.1 ± 3.9 (n = 4)	3.36 ± 0.17 (n = 63)	0.68 ± 0.10 (n = 63)	15 ± 1
LP1	5.3 ± 1.9* (n = 4)	2.56 ± 0.19* (n = 57)	0.44 ± 0.06* (n = 57)	14 ± 3
C2	38.0 ± 6.9 (n = 4)	3.38 ± 0.20 (n = 45)	0.65 ± 0.12 (n = 45)	11 ± 1
LP2	5.5 ± 6.0* (n = 4)	2.81 ± 0.34* (n = 51)	0.48 ± 0.10* (n = 51)	13 ± 2
C3	42.7 ± 2.0 (n = 6)	3.55 ± 0.20 (n = 87)	0.68 ± 0.12 (n = 87)	14 ± 2
LP3	7.1 ± 2.3* (n = 6)	2.81 ± 0.23* (n = 98)	0.54 ± 0.08* (n = 98)	14 ± 2

Maternal weight gain is expressed as percent of initial body weight for the average of animals in the feeding experiment. There was no significant difference in litter size throughout any experiment.

* $P < 0.005$ with respect to pair-fed controls

System A in C vesicles (Table 5-3) are similar to those of vesicles prepared from dams at the same gestational age fed chow *ad libitum*. Given that calorie-deprivation has been demonstrated to have a significant effect on amino acid uptake presumably mediated by System A (Rosso, 1975). Collectively, these data strongly support this model of maternal protein-deprivation with adequate calories.

Serum amino acid levels for LP dams were not lower than the levels for the C dams (Table 5-2). In fact, several amino acids were significantly increased. These data are similar to those obtained by other investigators for pregnant dams (Arola et al., 1992; Gomezangelats et al., 1995) and adult male animals (Swendseid et al., 1963; Colombo et al., 1992), fed a low-protein diet of similar composition to the diet fed in this study. Increased maternal serum amino acid levels seen in the LP animals are most likely a result of mobilization of nutrient stores by the catabolism of organs and skeletal muscle in that the wet weights of the kidneys, liver, and gastrocnemius muscle of the LP animals were significantly lower than those of C animals. The ratios of fetal to maternal (F/M) serum amino acid levels in the C animals were greater than one for nearly every amino acid tested (Table 5-2), suggesting concentration of these amino acids in the fetus. As nearly all amino acids are considered essential for the fetus and must be supplied by the maternal circulation (Mestyan and Soltez, 1984), these data illustrate concentrative delivery of amino acids to the fetus. However,

Table 5-2. Maternal serum amino acid levels from control and low-protein fed dams.

	CONTROL		LOW-PROTEIN	
	Maternal	Fetal	Maternal	Fetal
Alanine	278 ± 6	565 ± 109	1025 ± 281	627 ± 106
Asparagine	53 ± 13	70 ± 13	118 ± 27	78 ± 22
Aspartate	9 ± 2	32 ± 6	14 ± 3	30 ± 2
Cysteine	23 ± 3	28 ± 1	25 ± 4	30 ± 3
Glutamate	126 ± 9	323 ± 25	128 ± 13	267 ± 28
Glutamine	678 ± 32	639 ± 90	861 ± 30	661 ± 31
Glycine	154 ± 10	392 ± 62	557 ± 54	429 ± 18
Histidine	41 ± 6	60 ± 18	50 ± 11	26 ± 4
Isoleucine	67 ± 6	118 ± 13	74 ± 13	70 ± 1
Leucine	101 ± 2	206 ± 45	107 ± 17	140 ± 8
Lysine	596 ± 5	634 ± 78	695 ± 57	485 ± 16
Methionine	53 ± 5	125 ± 13	67 ± 17	84 ± 7
Phenylalanine	79 ± 8	187 ± 25	93 ± 21	178 ± 15
Proline	122 ± 22	166 ± 26	221 ± 26	153 ± 22
Serine	199 ± 42	307 ± 61	505 ± 48	323 ± 14
Taurine	171 ± 30	550 ± 46	218 ± 11	350 ± 6
Threonine	193 ± 1	247 ± 31	263 ± 6	173 ± 15
Tryptophan	40 ± 10	77 ± 7	48 ± 8	75 ± 8
Tyrosine	50 ± 4	187 ± 25	80 ± 9	108 ± 13
Valine	128 ± 19	250 ± 15	138 ± 21	176 ± 4

The maternal serum amino acid values are expressed in μmol/L. The data represent the means ± SD of 3 C dams and 3 LP dams.

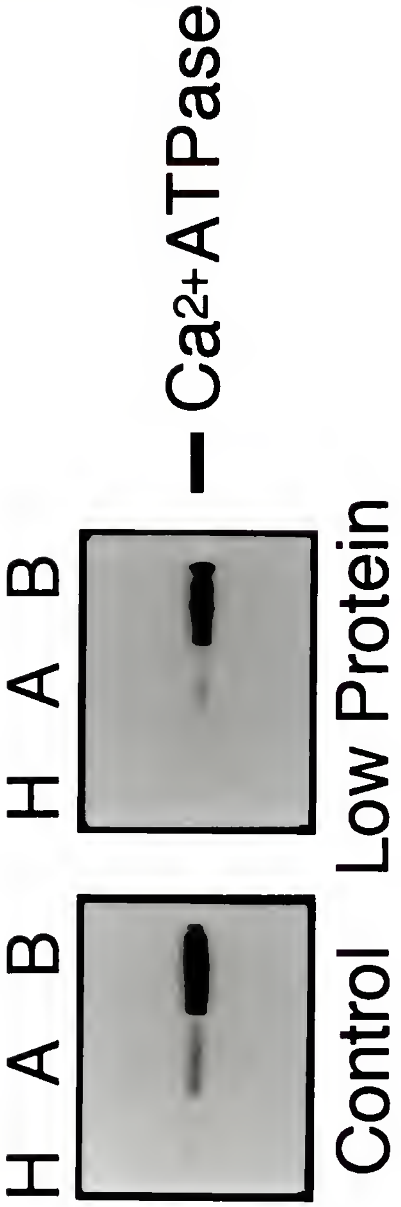
the LP fetuses had decreased levels of many amino acids even though the maternal pool of these nutrients did not change (Table 5-2: Ile, Leu, Met, Val, Arg, Tau, Glu). In some cases, even though there was an increased maternal serum amino acid level with the LP diet, the amino acid levels in the LP fetuses were significantly lower than the C fetuses (Table 5-2: Tyr, Thr, Lys). However, some amino acid levels were increased or unchanged in the LP fetuse reflecting the increased maternal levels. The levels of amino acids in the fetuses of LP animals are supported by previously published data from other investigators using maternal malnutrition as a model (Arola et al., 1982). Taken together, these data imply an effect of the low-protein diet on the placenta such that there is decreased transfer of nutrients from mother to fetus.

Characterization of apical and basal membrane vesicles. Alkaline phosphatase has been immunocytochemically localized to the apical membrane of rat trophoblast layer II (Glazier et al., 1993), the first functional barrier between the maternal circulation and the rat trophoblast cytoplasm. Therefore, we followed the enrichment of alkaline phosphatase as a marker for enrichment of the apical membrane preparation. Enrichments, with respect to homogenate, from either the C (41 ± 10) or LP (48 ± 12) preparations were not significantly different and were comparable to those obtained by our laboratory for apical membrane vesicles prepared from animals at day 20 gestation fed normal chow *ad libitum*

(Malandro et al., 1994). These values were also similar to or greater than those obtained for either rat or human placental apical membrane vesicles prepared by other investigators (Alonso-Torre et al., 1992; Mahendran et al., 1993).

Although no direct evidence exists with respect to the membrane localization of the ß-adrenergic receptor in the rat or human placenta, it is generally accepted that the receptor is localized to the basal membrane of the trophoblast (Boyd et al., 1979; Kelley et al., 1983). As such, the enrichment of ^{3}H-dihydroalprenolol (D^{3}HA) binding should follow enrichment of the basal membrane of rat trophoblast layer III, the last functional barrier between the maternal and fetal circulations. The enrichment of D^{3}HA binding with respect to the homogenate in C (22 ± 3) and LP (17 ± 4) membranes was not significantly different between the two groups and was comparable to enrichments obtained previously by our laboratory (Malandro et al., 1994). Furthermore, these values were comparable to those obtained by other investigators for rat and human preparations (Bravo et al., 1993; Marin et al., 1990). To further support the identity of the basal membrane preparation, we employed immunoblot analysis (Figure 5-1) of both the basal and apical membrane preparations with a monoclonal antibody against a Ca^{2+}ATPase which has been immunocytochemically localized to the basal membrane of human trophoblast and the basal membrane of rat trophoblast layer III (Borke et al., 1989). The basal

Figure 5-1. Immunoblot analysis of homogenate (H), apical (A), and basal (B) membrane vesicles from control and low-protein fed animals. An 50 µg aliquot of homogenate and 10 µg of either apical or basal membrane vesicles, was subjected to SDS-PAGE as described in Experimental Procedures. Immunoblot analysis was performed with a monoclonal antibody against the human placental Ca^{2+}ATPase. The blot shown is representative of three independent experiments.


H
A
B
H
A
B
Control
Low Protein
Ca2+ATPase

membrane preparation of both the C (14 ± 6-fold) and LP (13 ± 5-fold) was significantly higher in Ca^{2+}ATPase immunoreactivity over the apical membrane. These data demonstrate that the basal membrane vesicles arise from the basal membrane of trophoblast layer III, the membrane which serves as a functional barrier for solute uptake from and release into the fetal circulation.

Vesicle volume, as estimated from the steady-state distribution of ^{3}H-serine in the absence of Na^+, was not significantly different when comparing the C and LP groups (C apical 3.96 μl/mg protein and LP apical 3.93 μl/mg protein; C basal 3.33 μl/mg protein and LP basal 3.30 μl/mg protein).

Amino acid uptake in C and LP vesicles. To examine the effect of the low-protein diet on placental amino acid transport, representative neutral, anionic, and cationic amino acids were used as substrates for several distinct amino acid transport systems. MeAIB has been shown in all cell types tested to be a selective substrate for the uptake of amino acids through the Na^+-dependent System A (Kilberg et al., 1993); this activity has been localized to both the apical and basal membranes of the rat placenta (Alonso-Torre et al., 1992). To assay System A activity, uptake of 50 μM [^{3}H]-MeAIB was measured in both apical and basal membranes prepared from C and LP placentas. In a series of three independent experiments, Na^+-dependent MeAIB uptake was decreased on both the apical and basal membranes of placentas from LP dams with respect to pair-fed controls (Table 5-3).

Table 5-3. Transport of MeAIB (System A) in placental apical and basal membrane vesicles from control (C) and low-protein (LP) diet-fed animals.

	Na^+	K^+	Na^+-dependent	% Inhib.
			Apical	
C1	110 ± 0.8	26.9 ± 0.6	83.5 ± 1.0	
LP1	66.6 ± 1.8*	21.1 ± 1.7	45.5 ± 2.5*	49
C2	67.8 ± 4.8	18.2 ± 2.4	49.6 ± 5.4	
LP2	43.9 ± 2.6†	15.6 ± 1.9	28.3 ± 3.2†	38
C3	112 ± 0.9	35.6 ± 0.6	76.5 ± 1.1	
LP3	66.7 ± 3.4*	32.3 ± 3.3	34.4 ± 4.7*	55
			Basal	
C1	70.6 ± 0.3	27.9 ± 2.9	42.7 ± 2.9	
LP1	41.0 ± 1.8*	29.3 ± 1.1	11.7 ± 2.1*	73
C2	51.9 ± 4.0	23.6 ± 1.6	28.3 ± 4.3	
LP2	22.2 ± 1.4*	22.2 ± 1.6	0 ± 2.1*	96
C3	85.9 ± 0.2	40.5 ± 1.2	45.4 ± 1.2	
LP3	73.3 ± 1.6*	50.4 ± 1.6	22.9 ± 2.3*	56

The values are means (n = 3) ± SEM expressed as $pmol \cdot mg^{-1}$ $protein \cdot 10\ sec^{-1}$. Uptake of 50 μM $[^3H]$-MeAIB was assayed for 10 s at 37°C as described in Experimental Procedures. C1/LP1, C2/LP2, C3/LP3 represent three independent experimental trials.

* $P < 0.005$ with respect to pair-fed controls

† $P < 0.010$ with respect to pair-fed controls

The Na^+-independent uptake was not different between either group (Table 5-3). These data are in contrast to uptake of the neutral amino acid serine which, in the human placenta is primarily mediated by Na^+-dependent System ASC (Johnson and Smith, 1988). Uptake of 50 μM [^{3}H]-serine in the presence of inhibitors for Systems A and N, is consistent with System ASC activity and did not differ between C or LP preparations (C apical 11.9 ± 1.6 and LP apical 13.4 ± 2.0; C basal 21.5 ± 3.6 and LP basal 20.5 ± 1.8). These data also suggest that the decrease in amino acid transport is specific for individual transport systems rather than a general decrease resulting from a lack of materials for protein synthesis.

Transport of anionic amino acids occurs a variety of mammalian cells in a Na^+-dependent and Na^+-independent manner by Systems X^-_{AG} or x^-_c, respectively (Gazzola et al., 1981). In the human placenta, the only system expressed on both the apical and basal membranes is Na^+-dependent X^-_{AG} (Hoeltzli et al., 1989; Moe and Smith, 1990). Uptake through this system in the placenta has been shown to be dependent on an outwardly directed K^+ gradient (Iioka et al., 1988). Therefore, uptake of 50 μM [^{3}H]-glutamate was performed in vesicles preloaded with 100 mM KCl. Na^+-dependent uptake of glutamate in the apical membrane preparation did not significantly differ between C and LP groups at 20 sec, a time approximating initial rate (Na^+-dependent C 16.2 ± 3.5, Na^+-dependent LP 13.1 ± 0.8). However, in basal membranes, Table 5-4. Na^+-dependent transport of glutamate (System X^-_{AG}) in placental basal membrane vesicles from control (C) and low-protein (LP) diet-fed animals.

	Na^+-dependent	% Inhibition
C1	21.8 ± 3.3	
LP1	12.6 ± 4.2*	69
C2	18.9 ± 2.4	
LP2	11.0 ± 2.8*	53
C3	18.5 ± 1.9	
LP3	10.0 ± 2.7†	54

The values are means (n = 3-4) ± SEM expressed as $pmol \cdot mg^{-1}$ $protein \cdot 20\ sec^{-1}$. Uptake of 50 μM ^3H-glutamate was assayed for 20 s at 37°C as described in Experimental Procedures with the addition of a 100 mM KCl preloading of the vesicles and replacement of SCN^- with Cl^- in the uptake solutions at equimolar amounts. C1/LP1, C2/LP2, C3/LP3 represent three independent experimental trials.

* $P < 0.05$ with respect to pair-fed controls

† $P < 0.025$ with respect to pair-fed controls

initial rate uptake of glutamate is decreased over 50% in response to the low-protein diet (Table 5-4).

This study, assaying substrate specificity and competitive inhibition of transport, has previously characterized the rat placental complement of cationic amino acid transport systems on both the apical and basal membranes of rat placenta (Chapter 3). Transport of exclusively cationic amino acids occurs through the Na^+-independent System y^+, the primary route of cationic amino acid uptake in most fully-differentiated mammalian cells (White, 1985). Two transport systems capable of both cationic and small neutral amino acid uptake in the placenta, were first described in mouse blastocysts, Na^+-dependent System $B^{o,+}$ and Na^+-independent System $b^{o,+}$ (Van Winkle et al., 1985; Van Winkle et al., 1988). Consistent with previous findings, Na^+-dependent uptake of arginine (System $B^{o,+}$) was only detected in apical membrane vesicles. This activity was significantly decreased in response to the low-protein diet (Figure 5-2). System y^+ activity was detected in both membrane preparations and was reduced as well. The greatest effect was seen on System $b^{o,+}$, assayed as the leucine-sensitive component of arginine uptake. This activity was nearly completely abolished on the basal membrane of IUGR animals.

Northern analysis of CAT1 and EAAC1 mRNA levels. A cDNA probe (CAT1) for the System y^+ transporter (Kim et al., 1992), and a cDNA probe (EAAC1) for the System X^-_{AG} transporter (Velaz-Faircloth et al., in press) were used to correlate the

Figure 5-2. Na^+-dependent and Na^+-independent cationic amino acid uptake in apical and basal membrane vesicles from control (filled-bars) and low-protein (open-bars) fed animals. [^3H]-arginine uptake was assayed for 10 sec at 37°C as described in Experimental Procedures. Inhibitors, 2.5 mM leucine and 2.5 mM arginine (where indicated), were added to the uptake solutions to permit the selective assay of System $b^{o,+}$ and the non-saturable uptake component, respectively. Isoosmolarity was maintained at 310 mosmol with the addition of sucrose. Values are reported as means ± SEM for triplicate determinations. ** $P < 0.05$, * $P < 0.005$.

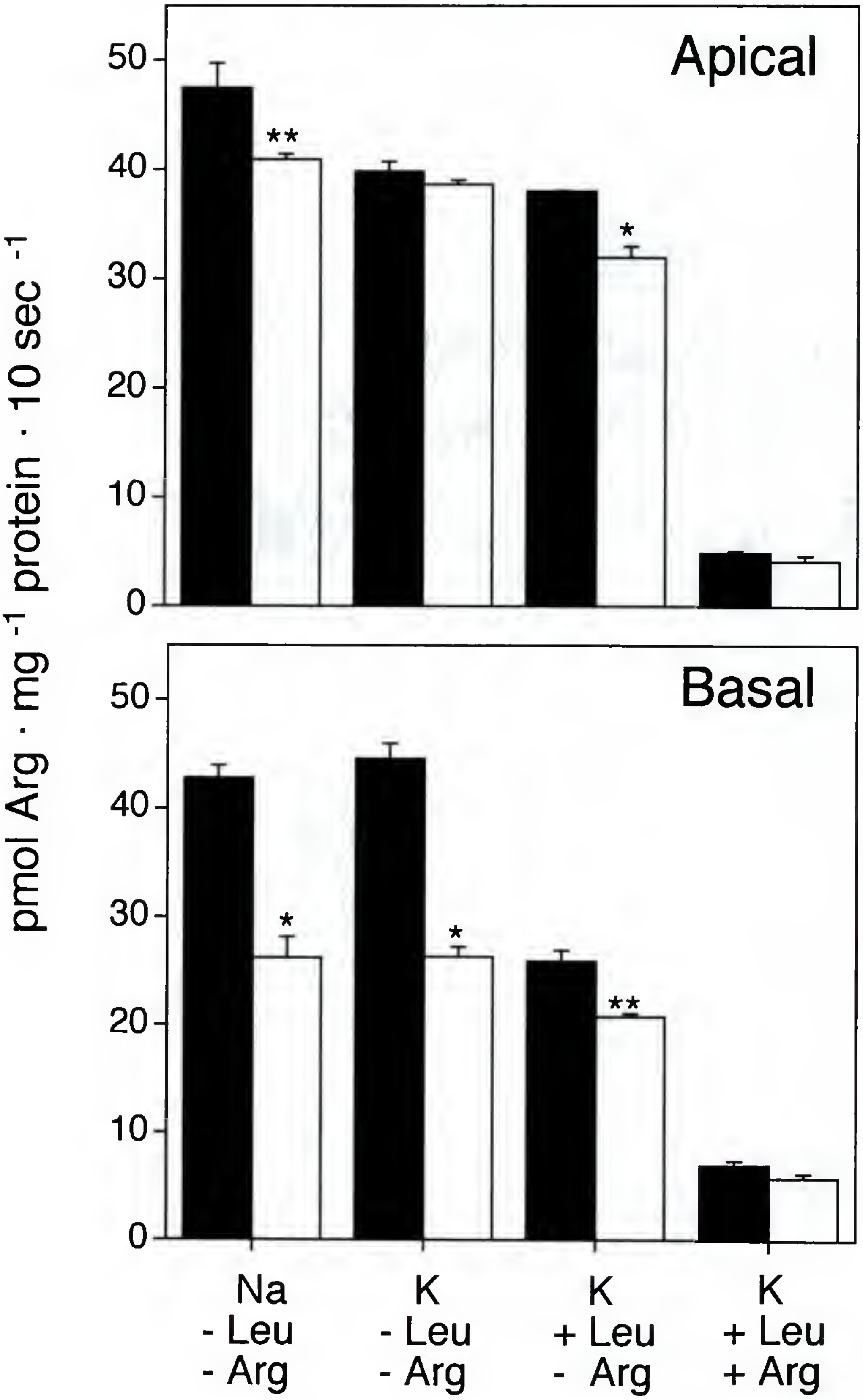
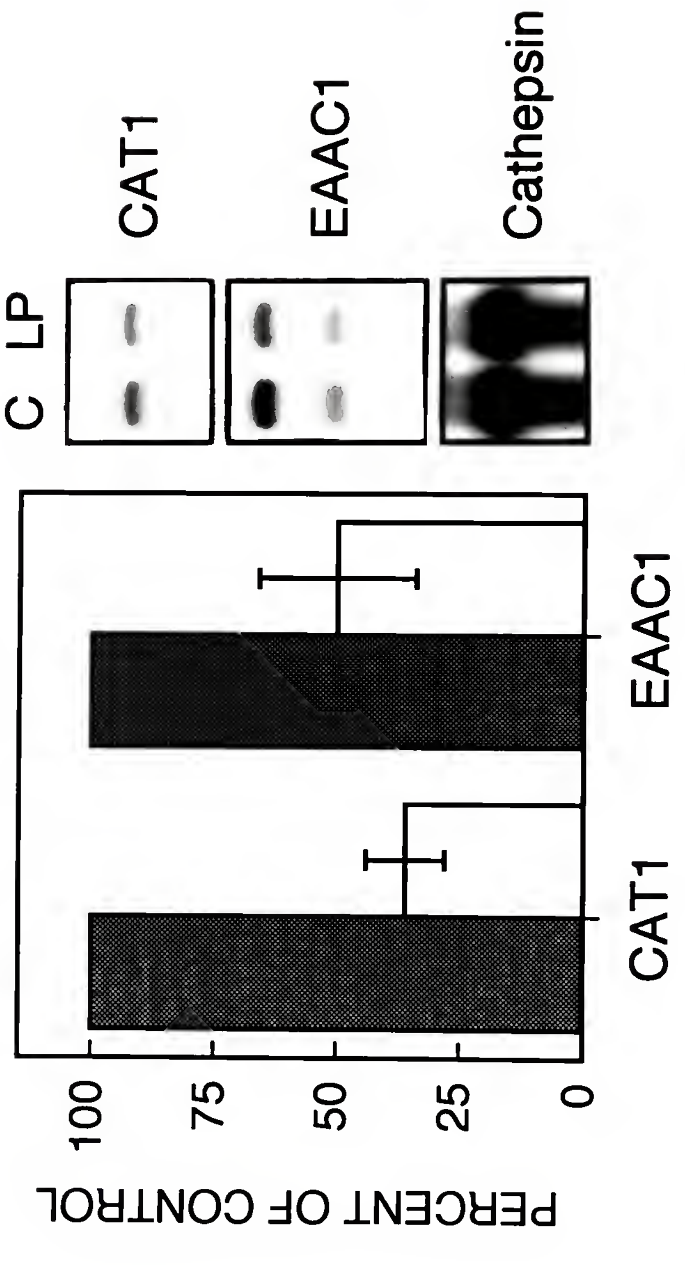

Apical
Basal
pmol Arg · mg -1 protein · 10 sec -1
0
10
20
30
40
50
**
*
*
*
**
Na
- Leu
- Arg
K
- Leu
- Arg
K
+ Leu
- Arg
K
+ Leu
+ Arg

Figure 5-3. Northern analysis of placental System y^+ (CAT1) and System X^-_{AG} (EAAC1) mRNA from control (closed-bars) and low-protein (open-bars) fed animals. Poly A^+ mRNA (3 μg per lane) was separated by 1% agarose gel electrophoresis and transferred to nylon membrane as described Experimental Procedures. The filter was hybridized with [^{32}P]-labeled cDNA probe and washed at 65°C (375 mM NaCl, 40 mM Na_2HPO_4, pH 7.2, 1 mM EDTA, 1% SDS). A cDNA probe to the constituatively-expressed cathepsin B mRNA was used to ensure equal RNA loading between lanes and used for normalization in the densitometric analysis. Densitometric data represent the mean ± SEM of three independent experimental trials. The data are presented as percent of control.


PERCENT OF CONTROL
100
75
50
25
0
CAT1
EAAC1
C
LP
CAT1
EAAC1
Cathepsin

steady-state cellular mRNA content with the transport activity in both the C and LP placentas. Consistent with previous analysis of CAT1 in rat placenta, a mRNA species of approximately 7.4 kb was detected in poly A^+ mRNA isolated from C and LP placentas. Densitometric analysis revealed a significant decrease in the steady-state content of System y^+ mRNA from placentas of low-protein fed dams (Figure 5-4). This result was not restricted to the cationic transporter. In fact, similar results were obtained following Northern analysis of the anionic amino acid transporter EAAC1 (4.0, 2.7, 2.2 kb). These mRNA data parallel the observed decrease in cationic (Figure 5-3) and anionic (Table 5-4) amino acid transport in both the apical and basal membrane vesicles prepared from low-protein fed dams.

Discussion

A majority of amino acids are essential for the developing fetus and as such, must be supplied by the maternal circulation (Mestyan and Soltez, 1984). Transporters on both the apical and basal membranes of the placental syncytiotrophoblast allow for the provision of these nutrients to the fetus from the maternal circulation. The current study demonstrates that specific down-regulation of amino acid transporters within both membrane domains in response to inadequate maternal nutrition decreases the available nutrient pool to the fetus and results in aberrant fetal growth.

The low-protein diet used in this study caused a marked reduction in maternal weight gain throughout gestation and resulted in significantly smaller fetuses. Previous investigators have examined the effect of diets with similar protein composition to the diet used in this study (Swendseid et al., 1963; Gomezangelats et al., 1995). However, in these studies, because the animals were not pair-fed, the low protein animals consumed less calories and as such, were calorie-deprived as well. These animals showed nearly the same decrease in maternal weight gain and decrease in fetal weight as seen in our study. This suggests that a decrease in dietary protein, even with the caloric deficiency compensated for by the addition of carbohydrate, is detrimental to fetal development. In sheep, the flux of amino acids to the fetus far exceeds the rate of nitrogen acretion into protein, it has been suggested that a considerable portion of the amino acid supply is utilized as an energy source (Van Vleen et al., 1989). Depletion of this energy source may contribute to many of the effects seen in this study. It should be noted that caloric deficency, as noted in studies of fasted dams, is also severely detrimental to the mother and fetus (Golde, 1989).

Analysis of the serum amino acid levels of the dams and fetuses demonstrate that most amino acids are concentrated in the fetal circulation in animals fed a diet adequate in protein. The fetal to maternal ratio (F/M) for some amino acids, including glutamate (2.6) and aspartate (3.6) is significantly higher in the rat fetus versus other species. The values for these two amino acids in particular are unique in that in the human and sheep, the F/M values for glutamate and aspartate are less than one indicating that the fetal levels are actually lower than the maternal levels for these amino acids (Marconi et al., 1989). For glutamate, it has been demonstrated in sheep that this amino acid is not transferred from mother to fetus but rather is involved in a metabolic cycle of nitrogen delivery to the fetus where glutamate, a product of glutamine deamination, is then extracted from the placental circulation by the trophoblast (Moores et al., 1994). As this study demonstrates a similar glutamate transporter complement as the human and sheep placentas (Hoeltzli et al., 1989; Marconi et al., 1989; Moe and Smith, 1990), the increased level of these amino acids in light of the glutamine/glutamate cycle remains to be described.

The observations included in this section suggest that the placentas of dams fed a protein-deprived diet have a reduced capacity to extract nutrients from the maternal circulation. These amino acids are then either metabolized by the trophoblast, or the decreased levels of these amino acids could result in a decreased flux to the fetus. A reduction in transfer could be accomplished in several ways. First, reduced maternal serum amino acid pools might simply be transferred proportionally to the fetus. Our observations show that animals fed the LP diet have serum amino acid levels that were either comparable to or increased when compared to those of pair-fed C animals. These data are not unique to this study in that pregnant dams pair-fed a diet of smiliar formulation showed an increase in some serum amino acids as well (Arola et al., 1982). Even though there was no reduction in the maternal serum amino acids, there was a significant decrease in the transfer of amino acids to the fetus. In some cases, even with higher serum amino acid levels in LP dams, the levels of these amino acids were decreased in the LP fetus compared to the C fetus. Several studies have demonstrated that suboptimal levels of even a single amino acid can impair normal development. These data have been presented for taurine, an amino acid reduced in the LP fetuses in this study (Sturman et al., 1988).

Another mechanism by which the decrease in transfer of amino acids might occur is by decreased availability of substrate to the transporters on the apical membrane. Several studies in humans and animals have shown that diminished uteroplacental blood flow occurs in association with IUGR (Bracero et al., 1989). In fact, an observed decrease in glucose flux in calorie-deprived rats is associated solely with a decrease in placental blood supply (Jansson et al., 1993). When those investigators examined protein levels and activity in placental apical membrane vesicles, there was no significant decrease between control and calorie-deprived groups. In contrast to those results for glucose, the analysis of isolated vesicles described in this section show a significant decrease in amino acid transport. Any effects of decreased blood supply on nutrient flux would be additive to the transporter specific effects and would, therefore, further diminish fetal amino acid supplies. One should also consider the decrease in placental size that was noted in the low-protein diet fed dams the decrease in nutrient accessible surface area. This effect as well would serve to further contribute to the IUGR condition.

Following protein-deprivation, there is a parallel decrease in transport activity and placental mRNA content for several transporters. Until techniques are available to isolate cell specific nuclei from the placental syncytiotrophoblast, one cannot determine if the mRNA loss is due to a decreased rate of transcription or increased mRNA turnover. The reduction of transporter mRNA strongly suggests a reduction in the synthesis of the corresponding membrane transport proteins rather than a secondary effect on existing proteins.

The mechanisms that function to sense maternal amino acid availability and transfer this information to transporter gene expression in the placenta are unknown at present. However, amino acid-dependent changes in gene expression have been documented for a number of proteins, including amino acid transporters (Kilberg et al., 1993). The lack of significant decreases in circulating maternal amino acids would seem to preclude a direct signalling to the syncytiotrophoblast. Other potential regulatory mechanisms, hormone signalling in particular, must be considered. Regardless of the mechanism, our data show that changes in maternal nutrition are responsible for control of placental amino acid transport by regulating the level of transporter mRNA content. The cloning of an increased number of transporter cDNAs and identification of the corresponding transport proteins will permit future experiments to define these regulatory mechanisms in greater detail.

CHAPTER 6
DEVELOPMENT OF A STABLE MAMMALIAN EXPRESSION SYSTEM FOR THE CLONING OF AMINO ACID TRANSPORTERS

Introduction

Since 1991, considerable progress has been made toward the identification of cDNA clones responsible for charged amino acid transport; however, many activities, especially with regard to neutral amino acid transport, remain to be described. The most highly regulated of these transport activities, and as such the transporter for which a cDNA could be the most useful, is System A.

System A is present in every nucleated mammalian cell tested and mediates the Na^+-dependent uptake of small neutral amino acids with no branching on the ß-carbon (Kilberg, 1986). Non-metabolizable, model substrates have been described for System A in AIB and MeAIB (Christensen, 1982; Collarini and Oxender, 1981). System A has been shown to be induced by 1) glucagon treatment (Kilberg et al., 1985); 2) streptozotocin-induced diabetes (Barber et al., 1982; Handlogten and Kilberg, 1984); 3) cell transformation (Saier et al., 1988); 4) substrate starvation (Shotwell et al., 1983); and 5) cytokine and growth factor treatment (Collarini and Oxender, 1987; Saier et al., 1988). It should be noted that the regulation of System A in the above examples has been assayed by measurement of transport activity alone. Further analysis of the regulation of System A must come from measurement of mRNA and protein levels to complement the vast literature of activity studies.

Although only activity has been used to follow System A, several important observations have been made. The induction of System A, regardless of stimulus, has been shown to be completely blocked by the addition of actinomycin D and cycloheximide suggesting that induction of System A requires the synthesis of mRNA and protein, respectively (Pariza et al., 1976; Christensen and Kilberg, 1987). In fact, injection of Xenopus oocytes with mRNA from control and glucagon-induced cells demonstrates increased System A activity associated with the glucagon-induced mRNA suggesting that increased transcription is associated with the increased transport activity (Tarnuzzer et al., 1990). The protein required for glucagon-induction of System A is most likely a trans-membrane glycoprotein based on the following observations. First, the glucagon-induced activity can be measured in isolated plasma membrane vesicles (Schenerman and Kilberg, 1986). Although this activity cannot be stripped from the membrane with salt or low detergent concentrations, it can be isolated from the membrane with cholate/urea and reconstituted into proteoliposomes to yield activity proportional to intact membrane vesicles (Bracy et al., 1987). The glucagon-induced activity is also completely blocked by tunicamycin, an inhibitor of glycoprotein biosynthesis (Barber et al., 1983). These data taken together suggest that induction of System A requires increased transcription and synthesis of a membrane-bound glycoprotein that is either the transporter itself or a membrane-bound regulatory subunit. As described in Chapter 1, none of the Na^+-dependent or Na^+-independent amino acid transporters isolated to date are multi-subunit complexes, these experiments have proceeded from the assumption that the induced activity represents increased synthesis of the transporter protein itself. However, the methodology described herein is equally applicable to the cloning of multi-subunit transporters as well.

In spite of the fact that several laboratories have demonstrated the expression of neutral amino acid transport activity in mRNA injected Xenopus oocytes (Tate et al., 1989; Palacin et al., 1990; Tarnuzzer et al., 1990), the cDNA for System A has yet to be isolated. Oocyte expression cloning has proved useful for the isolation of ion exchangers (Hirono et al., 1985; Sigel, 1987) and the Na^+-dependent glucose transporter (Hediger et al., 1987a). However, the only two proteins related to α-amino acid transport to be isolated by oocyte expression cloning are the rBAT protein, which may more likely be inducing an already present oocyte transporter rather than mediating amino acid uptake through a protein product of its own coding sequence, and the Na^+-dependent glutamate transporter EAAC1. For example, even though there are a number of kinetically distinguishable amino acid transport activities observed in the kidney, the rBAT cDNA remains the only amino acid transport-associated protein isolated by this approach.

This chapter describes a novel approach for cloning amino acid transporters centered around a mammalian cell expression system which permits isolation of expressing cDNAs in episomally-maintained prokaryotic shuttle vectors (Margolskee et al., 1988; Swirski et al., 1992). Increased transport mediated by specific clones in an expression library are detected on replica filters allowing for isolation of viable colonies corresponding to those colonies demonstrating increased transport activity. The efficacy of these procedures was tested by use of a control amino acid transporter cDNA, the rat EAAC1 Na^+-dependent glutamate transporter cDNA described in Chapter 3.

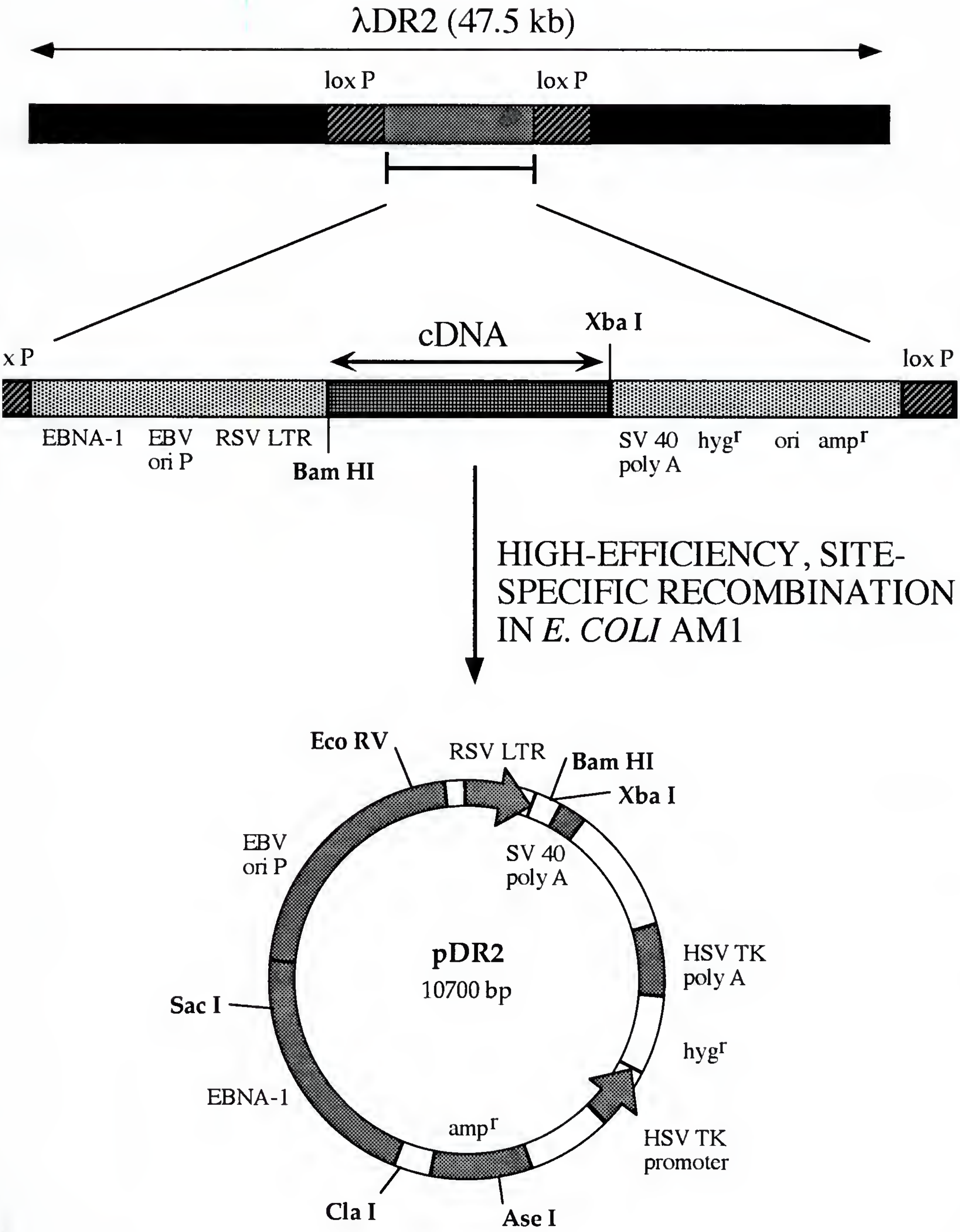
Methods

Materials. Vectors (pDR2, λDR2), cell lines (293c18), *E. coli* strains (K802,AM1), and primers were the generous gift of Dr. Robert Schimke at Stanford University. Subsequently, the materials have been made commercially available from Clonetech Laboratories (Palo Alto, CA). All general protocols with respect to cell maintainence and growth were provided by Clonetech. Polyester filters (HD7-1, 9 μm) were obtained from Tetko (Elmsford, NJ). Lipofectamine reagent was obtained from GibcoBRL (Gaithersburg, MD). All other materials were of the highest quality available from vendors listed in Chapter 2.

Maintainence of λDR2 and conversion to pDR2. The pDR2 plasmid vector is completely contained within the λDR2 bacteriophage vector (Figure 6.1) and can be isolated by infection of a recombinase-positive *E. coli* strain (AM1). A 15 ml culture of LB broth is innoculated with single colony of AM1 and grown overnight at 32°C. The cells were pelleted by centrifugation and resuspended in 10 mM $MgSO_4$. A 100 µl aliquot of cell suspension was infected with 5 x 10^7 PFU λDR2 vector containing the rat EAAC1 cDNA (Chapter 3) for 30 min at 32°C. The entire mixture was then added to 10 ml 2x YT broth and incubated for 1 hr at 32°C. 50µl 100 mM IPTG, 50 µl 50 mg/ml carbenicillin, and 200 µl 1M sodium citrate was added to the culture, and the culture incubated for another two hrs at 32°C. The culture was then divided into six parts and added to six 1000 ml aliquots of pre-warmed (37°C) LB broth. The cultures were then incubated at 37°C overnight with vigorous shaking. The switch in temperature from 32°C to 37°C activates the temperature sensitive recombinase such that the infected AM1 cells are able to "pop out" the pDR2 plasmid by homologous recombination at the two *lox p* sites flanking the pDR2 vector (Figure 6.1). The EAAC1 cDNA in the pDR2 plasmid vector was then isolated by a well established alkaline lysis protocol (Sambrook et al., 1989).

Transfection of 293c18 cells with pDR2. Freshly thawed 293c18 cells in low passage were incubated in DMEM

Figure 6-1. Diagram and vector map of λDR2/pDR2. The pDR2 plasmid is contained within the two lox P sites of the 47.5 kb λDR2 vector. cDNAs are cloned between the BamHI and XbaI sites of pDR2. Restriction endonuclease sites are shown in bold type. EBNA = Epstein-Barr nuclear antigen, EBV oriP = Epstein-Barr virus origin of replication, HSV TK/SV40 poly A = herpes virus thymidine kinase/Simian virus 40 polyadenylation site, amp^r/hyg^r = ampicillin/hygromycin resistance gene, ori = E. coli origin of replication, RSV LTR = Rous sarcoma virus long terminal repeat, HSV TK = herpes simplex virus thymidine kinase promoter.


λDR2 (47.5 kb)
lox P
lox P
cDNA
Xba I
x P
lox P
EBNA-1
EBV ori P
RSV LTR
Bam HI
SV 40 poly A
hygr
ori
ampr
HIGH-EFFICIENCY, SITE-SPECIFIC RECOMBINATION IN E. COLI AM1
Eco RV
RSV LTR
Bam HI
Xba I
EBV ori P
SV 40 poly A
pDR2
10700 bp
HSV TK poly A
Sac I
hygr
EBNA-1
ampr
HSV TK promoter
Cla I
Ase I

supplemented with 10% FBS at 37°C in 5% CO_2 until just subconfluent (80%). Cells were then transfected by liposome-mediated transfection with Lipofectamine reagent using protocols established by the vendor (GibcoBRL, Gathersburg, MD), and included 4 μg DNA, 12 μl Lipofectamine per 1 ml reduced-serum medium (OptiMEM, GibcoBRL). The liposome/DNA mixture was left on the cells for six hrs, then replaced with DMEM/FBS. Cells were allowed to recover for 24 hrs before the addition of hygromycin B at 250 μg/ml in DMEM/FBS. Selection proceeded for 10 days in which the medium was changed every three days. After 10 days, the cells were washed 3 times with PBS (pH 7.4), trypsinized, and resuspended in DMEM/FBS. The cells were counted, and an aliquot frozen in liquid N2 in DMEM/FBS supplemented with 10% dimethyl sulfoxide. The remainder of the culture was plated as described below.

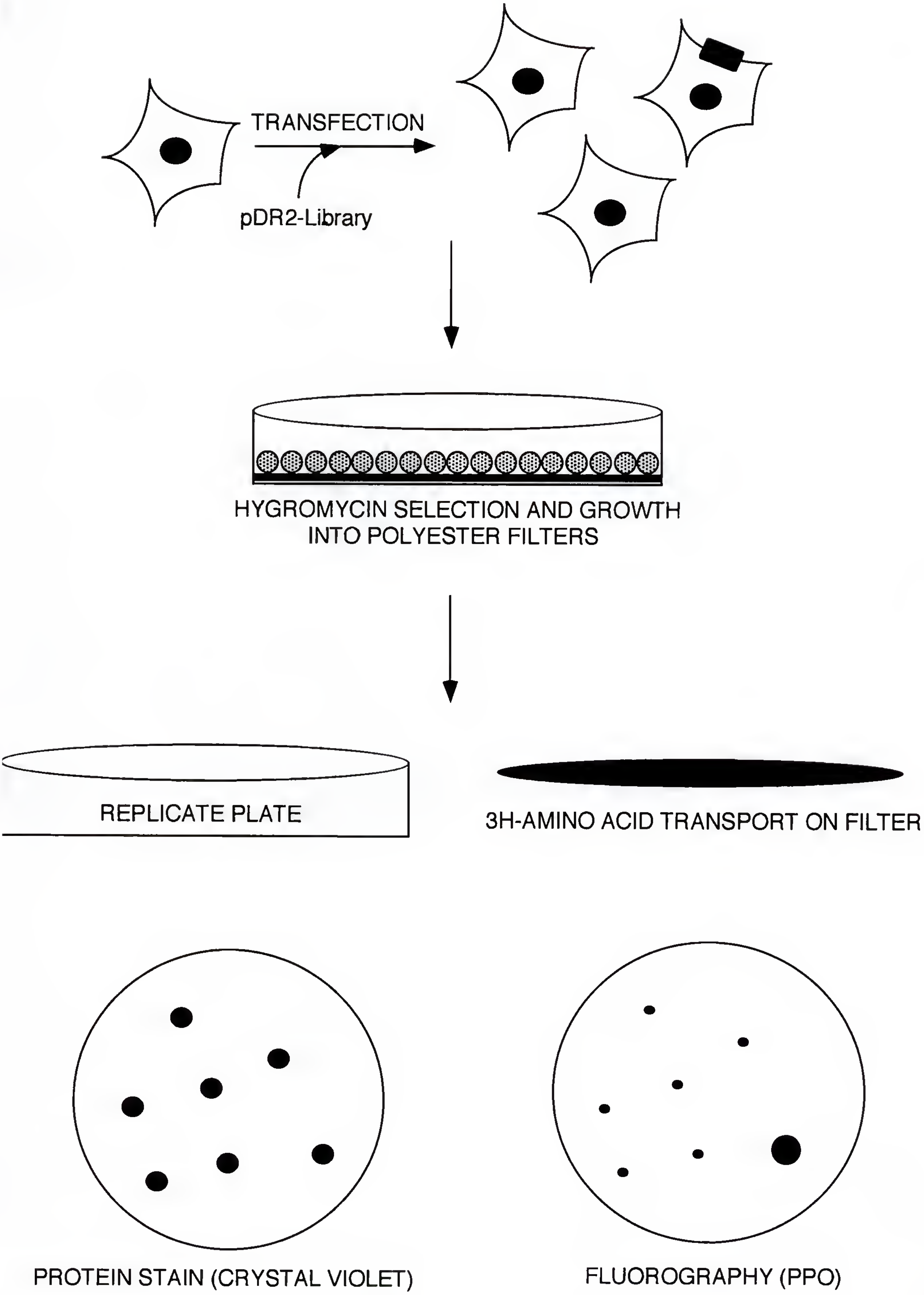
Plating and screening of transfected 293c18 cells. A mixed culture of 10% EAAC1-transfected 293c18 cells and 90% vector-transfected cells were plated at a density of 10,000-20,000 per 150 mm culture plate in DMEM/FBS supplemented with 250 μg/ml hygromycin B. The polyester filter culture conditions have been published previously (Esko, 1989; Dantzig et al., 1982). All plates were pre-coated with 0.01 mg/ml poly-L-lysine in PBS for 1 hr. The cells were incubated for 24 hrs before overlaying the culture with up to five polyester filters (#HD7-1, 9 μm, Tekto Inc.). The filters were subsequently overlayed with 4 mm glass beads to keep the filters in place and to promote the growth of the cell culture into the filters rather than spreading on the plate. The cells are incubated for 10 days, with medium changes every three days, to allow for cell growth into the filters. After 10 days, the filters and plates were marked such that individual colonies with increased transport will correspond to viable colonies on the plate. Radiolabelled substrate, 5 μM [^{3}H]-glutamate, not in tracer quantities but all radioactive, was added to 37°C Na$^+$KRP, and uptake assayed for 2.5 min. The filters were then washed three times in large volumes of ice-cold Na$^+$KRP and dipped into 10% PPO in acetone. The filters air-dried quickly and were exposed to pre-flashed autoradiographic film overnight. The filters were then stained the next day in 0.2% crystal violet in water to stain for total protein.

Results

The pDR2 vector (Figure 6.1) has many features that make it uniquely suited to expression cloning. First, when transfected into permissive cell lines expressing EBNA-1 (Epstein-Barr virus transforming protein), the vector is maintained episomally through several passages. Thus, under appropiate selection, in this case hygromycin B resistance, expression of cDNAs from pDR2 resembles stably-transfected cells. The major difference is that the pDR2 vector does not incorporate into the host cell genome, facilitating its isolation. This vector is also a shuttle vector in that it contains an *E. coli* origin of replication and ampicillin-resistance gene for direct transformation into bacterial cells. The expression of cDNAs is mediated by a RSV promoter and results in high levels of expression as is evidenced by the 40-fold induction of 50 μM [^{3}H]-glutamate uptake in 293c18 cells expressing the rat EAAC1 cDNA (61.1 ± 4.6 pmol·mg^{-1} protein·min^{-1} versus 2396.1 ± 209 pmol·mg^{-1} protein·min^{-1}). The lack of integration of the pDR2 vector results in the loss of cDNA expression after multiple passages. However, a 22-fold induction of [^{3}H]-glutamate transport is still seen after six passages (66.5 ± 2.9 pmol·mg^{-1} protein·min^{-1} versus 1463.6 ± 152 pmol·mg^{-1} protein·min^{-1}), much longer than the estimated time required for isolation of a potentially positive cDNA.

The major limitation of previous amino acid transporter cloning procedures was not the expression of the activity, as is evidenced by the expression of System A transport activity in Xenopus oocytes, but the limitation rather, lies in the ability to recover the cDNA responsible for the activity. The capacity of the pDR2 vector to be recovered as a plasmid with the ability to grow in E. coli overcomes this limitation. First, however, one must be able to identify and isolate a viable colony with the expressed cDNA. To this end, the stably transfected 293c18/EAAC1 cell line was used a positive control for the analysis of a somatic cell polyester filter-lift procedure (Figure 6.2)(Esko, 1989; Dantzig et al., 1982). In this procedure, cells are grown on poly-L-

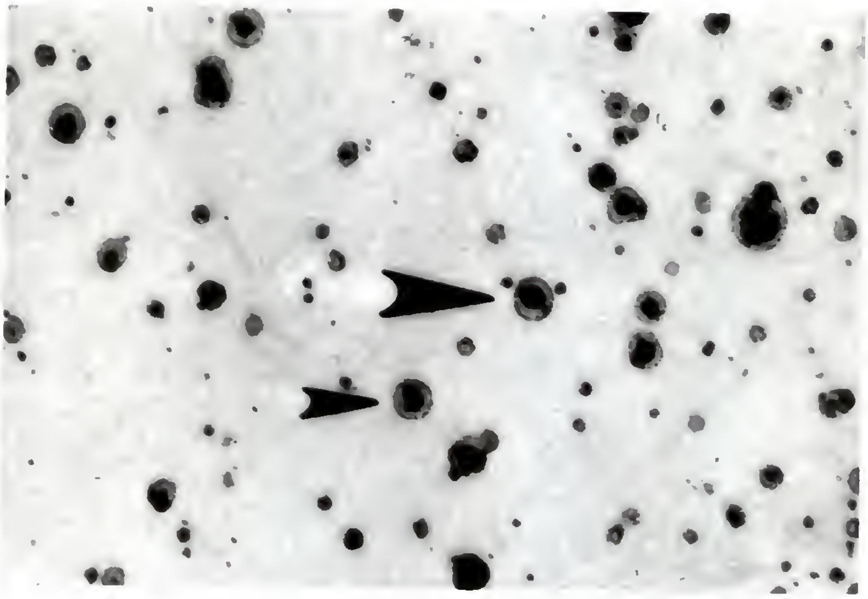
Figure 6-2. Schematic diagram of library screening protocol. Individual steps are described in the text.


TRANSFECTION
pDR2-Library
HYGROMYCIN SELECTION AND GROWTH INTO POLYESTER FILTERS
REPLICATE PLATE
3H-AMINO ACID TRANSPORT ON FILTER
PROTEIN STAIN (CRYSTAL VIOLET)
FLUOROGRAPHY (PPO)

lysine coated 150 mm culture dished overlayed with up to 5 layers of 9 μm polyester mesh. The mesh is then covered with 4 mm glass beads to inhibit the spreading of the growing cells. Instead, the cells grow into the mesh and form discrete colonies which can be visualized on the filter by crystal violet staining (Figure 6.3). A population of 1:10 EAAC1-transfected to vector-transfected cells were grown into the polyester mesh for 10 days. The filter-bound cells were then assayed for the uptake of 50 μM [^{3}H]-glutamate as was described previously by other investigators for the detection of aspartate transport mutant mammalian cells (Ash et al., 1993). Cells were incubated for 1 minute in the [^{3}H]-labelled amino acid, washed three times in cold buffer, and finally dipped in 15 % PPO in acetone. The colonies were then detected by overnight exposure to pre-flashed autoradiographic film. As can be seen in Figure 6.3, all cells show some degree of background uptake consistent with the value reported above for the control uptake of 50 μM [^{3}H]-glutamate. However, comparison of the level of protein from the stained filter with the level of glutamate transport from the autoradiogram, the EAAC1-transfected cells show significantly increased transport per unit of protein.

As the filters are removed for the analysis of transport activity described above, the cation-coating of the culture plate retains a portion of the cell colony essentially forming a "replicate pattern" of the filter-bound cells. One can identify a particular colony with increased transport

Figure 6-3. Crystal violet stain (left panel) and autoradiograph (right panel) of a representative screening with EAAC1-transfected 293c18 cells. Cells were grown into polyester filters for 10 days as described in the text. Transport of 5 μM [^{3}H]-glutamate was carried out for 3 min at 37°C. Bound radioactivity was detected by dipping the filters in 10% (v/v) PPO in acetone and then exposing the filters to film. Filters were then stained with 0.1% (w/v) crystal violet to detect cellular protein. Arrows indicate a positive and negative colony for comparison of protein and transport.



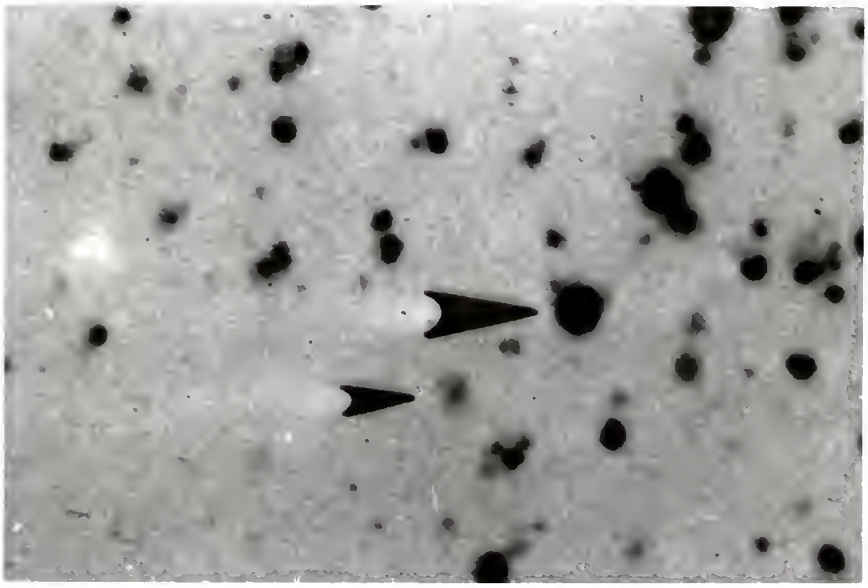
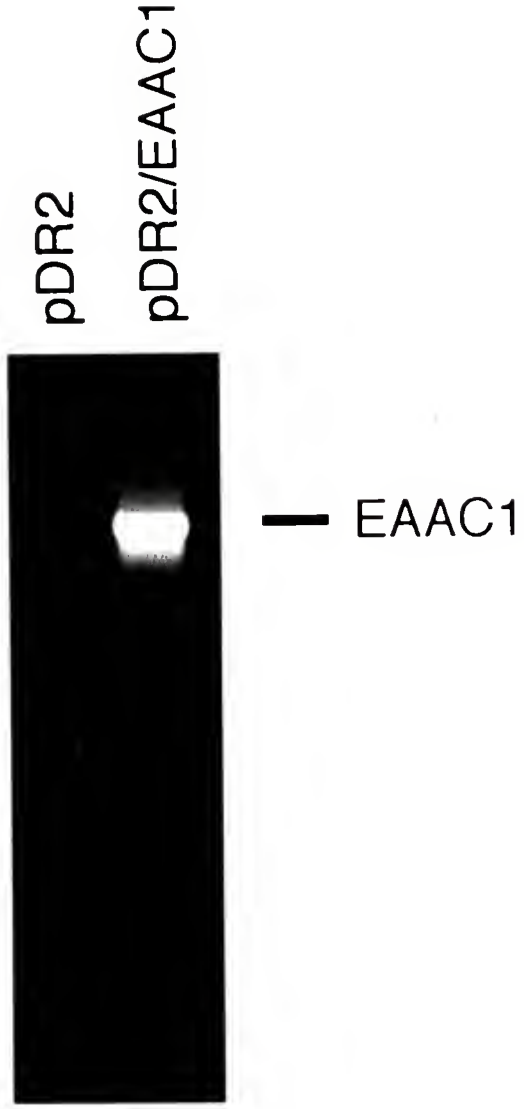


Figure 6-4. PCR detection of EAAC1 from a positive (pDR/EAAC1) 293c18 colony. A single colony isolated from the filter screening in Figure 6-3 was grown to confluency and the episomal DNA subsequently isolated. An aliquot of the extracted DNA was subjected to 2 rounds of PCR, the first round with primers specific to pDR2 vector flanking the cDNA, the second round with primers specific to rat EAAC1. A colony transfected with pDR2 alone was used as a negative control.


pDR2
pDR2/EAAC1
EAAC1

activity on the filter and match that colony with an intact cell population on the master plate, "pick" the positive colony by either local trypsinization or cloning loop, and by virtue of the hygromycin B selectable marker on the pDR2 vector, obtain a pure culture expressing the cDNA of interest. The cDNA can then be "rescued" by standard molecular biological methods. The colony identified in Figure 3 with increased glutamate uptake was subcultured in a 100 mm tissue culture plate in the presence of 250 μg/ml hygromycin B until confluence. The pDR2 vector was then isolated from the expressing cells by an alkaline lysis procedure (Sambrook et al., 1989) and analyzed by PCR for the presence of the EAAC1 cDNA. Oligonucleotide primers corresponding to flanking sequences on either side of the pDR2 multiple cloning site were used to PCR from the vector obtained from the cell culture, the original vector used to transfect the cell culture, and vector with no cDNA. As can be seen in Figure 6.4, products with a molecular size corresponding to the EAAC1 cDNA (2.2 kb) were seen when the vector isolated from the positive cells and the original pDR2/EAAC1 vector were used as the template. These data collectively demonstrate the effectiveness of our novel cloning approach to the isolation of amino acid transporter cDNAs.

Discussion

In the past several years, the field of amino acid transport has been advanced by the introduction of molecular biological tools made possible by the cloning of several families of amino acid transporters (Chapter 1). The pattern that has emerged from this cloning is that a single cDNA was isolated, either by concerted effort or serendipitous identification, and other families or related clones were then identified by low-stringency library screening or PCR. Although this has proven useful for the isolation of several cDNAs (ASCT1, PROT), it is limited in that the basis for cDNA identification by these techniques requires the availability of sequence information. For the case of transport activities where no sequence information exists, these techniques are generally not applicable.

Other techniques have been used for the identification of membrane proteins, but these have not proven useful for the identification of amino acid transporter cDNAs. *Xenopus* oocyte expression cloning has been used succesfully for the identification of ion exchangers (Hirono et al., 1985), ion channels (Sigel, 1987), and the Na^+-dependent glucose transporter of intestine and kidney (Hediger et al., 1987). However, the only classes of proteins related to amino acid transport to be isolated by this method are the rBAT/4F2hc family whose function is more likely inducing an already present amino acid transporter (Palacin, 1994) and the Na^+-dependent glutamate transporter EAAC1 (Kanai and Hediger, 1992). Several laboratories have expressed amino acid transport activities in oocytes using fractionated tissue mRNA, but these have not lead to the identification of the corresponding cDNAs. Protein purification and subsequent microsequencing of these isolated proteins has only been marginally successfull in isolating transporters. This may be due to the relatively low abundance of these transport proteins but more likely may be the result of association of transport activities with other more abundant proteins. This is the case for System A activity in that the interaction of transport activity with a cytoskeletal protein (fodrin) is so strong that 2.5% detergent and 4 M urea is required to solubilize the transporter. This interaction lead to the identification of fodrin by both microsequencing and antibody recognition. It is clear that novel approaches are required for isolation of transporter proteins.

The pDR2 filter lift screening system described in this chapter overcomes many of the limitations associated with other cloning techniques. First, this system allows for the screening of libraries by activity assay alone. Thus, the isolation of transport activities unrelated to any previously cloned cDNAs is possible with this system. This is not the case for λ libraries where screening with labelled cDNA or antibody requires some information about the protein of interest. In our laboratory, subtractive hybridization of enriched cDNA libraries in λgt11 was used in an attempt to isolate the transport protein associated with System A activity (Shay et al., 1990). Although this approach resulted in the isolation of several amino acid starvation induced mRNAs, it was unsucessful in the isolation of transport System A.

The pDR2 system also allows for the direct isolation of the cDNA responsible for the expressed transport activity. This is a limitation associated with Xenopus oocyte expression cloning. Although transport activity can be expressed in these cells, there is no methodology available for the isolation of mRNAs after analysis of transport activity. Oocytes do not efficiently transcribe mRNA from cDNA because these cells at this stage have accumulated the total mRNA complement necessary for development until fertilization (Hediger, 1994). There is no need in oocytes for transcription of mRNA, and as such, cRNA must be in vitro transcribed and injected rather than injecting a plasmid vector that would permit subsequent isolation. The shuttle vector pDR2 allows for direct transformation into bacteria without any other cloning steps, and in the case of extremely low abundance cDNAs, pDR2 has PCR primers flanking the cDNA for amplification and subsequent cloning. The ability of this vector to be maintained as an episome also alleviates the complications associated with other stable transfection systems. The random incorporation of vector DNA requires considerable effort for removal, identification, and subcloning.

The pDR2 system is not without some limitations. First, as is the case with any expression library, expression of transport activity most likely requires full-length cDNAs. The average insert size of commercially available libraries in pDR2 is 1.7 kb (Clonetech). The amino acid transporter cDNAs isolated have an average coding length of 1.9 kb (Chapter 1). Thus, the quality of the library in terms of abundance of long mRNAs is critical in limiting the number of rounds of screening of the library.

Another, and perhaps the most serious limitation, is inherent to the transporter cDNAs rather than a problem with the approach. While the expression of EAAC1 in mammalian cells results in a 20- to 40-fold increase in transport activity, the expression of ASCT1 only results in a 2-fold increase compared to vector-transfected controls (Tamarappoo et al., 1996). The reason for this low activity is unclear because other transporter cDNAs (PROT) expressed in mammalian cells have significantly higher activities. If the low activity of expressed ASCT1 held for the pDR2 system, its identification by this technique may be difficult. However, the methodology described in this chapter represents a novel approach for the identification of transporter cDNAs.

CHAPTER 7
CONCLUSIONS AND FUTURE DIRECTIONS

For several decades it has been known that the maternal circulation provides nutrients to the fetus, but the role of the placenta in providing these nutrients still is not fully appreciated. This study and others of its kind are beginning to go further than simply defining the nutrient transporters present at the syncytiotrophoblast interface by examining the regulation of these transporters during normal and aberrant development.

First, contrary to the former belief that all Na^+-dependent transporters are present on the apical membrane to extract nutrients from the maternal circulation, Na^+-dependent transporters also exist on the basal membrane to transport amino acids back to the trophoblast. These transporters play a role in specalized metabolic cycles for delivery of nitrogen and nutrients from the mother to fetus and back. This study is also the first to define by immunologic methods the presence of a specific amino acid transporter protein at either membrane surface. This represents a tremendous advance to the field, in that before this work, all analysis of amino acid transport activity in the placenta was done on an activity basis alone. With so many the transport systems having overlapping substrate specificities and ion dependence, kinetic analysis in a tissue where multiple transporters are present is difficult. Molecular analysis, however, should not replace kinetic studies of transport activity but rather complement these studies. One should remember that the ultimate goal is the understanding of nutrient passage across the membrane, and as such, the physical measurement of this activity will always be necessary.

Second, during the latter third of gestation, the time in which the fetus is at its greatest nutritional need, amino acid transport is increased in both delivery of essential substrates and removal of metabolites from the fetal circulation. Furthermore, this increase in transport is not merely a result of increased concentrations of these substrates in the maternal or fetal circulation, but rather result from increased synthesis of amino acid transport proteins at both the apical and basal membrane surfaces in the placenta.

Third, altering the nutrient supply, in this case by decreasing protein to the mother, is detrimental to the fetus. This contradicts the Hammond Hypothesis which states that the fetus will extract nutrients to meet its needs at the expense of the mother. If this were the case, then there would be no decrease in fetal or placental size associated with moderate nutritional inadequacies. This study also demonstrates that the down-regulation of these transporters is not simply a result of decreased maternal pools being passed on to the fetus because, the maternal serum amino acid levels were not significantly decreased in undernourished animals. Also, the decrease in transport activity was associated with an decrease in steady-state mRNA as well.

In a study such as this, one would like to establish cause and effect relationships between the decrease in amino acid transport, and the presence of small for gestational age (SGA) fetuses. While the data presented here do not contradict this relationship, they do not definitively prove it either. More likely is a complex relationship between nutrient availability and fetal and maternal hormane production. A decrease in maternal dietary protein intake has been shown to decrease the circulating insulin in the fetus (Golde, 1989). Insulin, in addition to its role in glucose homeostasis, in the fetus serves as a potent growth factor, and low levels of insulin production is associated with SGA pregnancies (Sips et al., 1982). Thus, the decrease in the protein content of the LP diet may be not only detrimental to the fetus by lack of essential nutrients, but may also have an effect on growth factor release together resulting in smaller fetuses.

Extensions of this study would focus on the available molecular tools to examine transporter protein and mRNA. Clearly, there is a finite number of cDNAs related in sequence to those that have been previously cloned, and as such, techniques such as low stringency PCR and hybridization are limited to that small set of proteins. A great need exists for a cloning system that allows for the isolation of cDNAs based on amino acid transport activity alone. At the present time, oocyte expression cloning has not filled that need. The efficacy of the pDR2 library screening system has been demonstrated by detailed analysis of each step with control cDNAs. However, the apparently low abundance of aminoid transporter mRNAs in some cases will truly put this system to the test and require several round of screening to identify transport-related cDNAs.

Taken together, the data presented in this manuscript represent an effort to utilize available molecular and biochemical tools to examine amino acid transport during normal and abnormal development of the fetus and to develop new approaches for generating these tools where they are lacking.

LIST OF REFERENCES

Ahkoas, R. A., E. B. Lahyae, G. D. Anderson, and J. Lipshitz. J. Nutr. 111: 2052-2058, 1981.

Albritton, L. M., L. Tseng, D. Scadden, and J. M. Cunningham Cell 57:659-666, 1989.

Alonso-Torre, S. R., M. A. Serrano, J. M. Medina, and F. Alvarado. Biochem. J. 288:47-53, 1992.

Arola, L., A. Palou, X. Remesar, and M. Alemany. Horm. Metab. Res. 14:364-371, 1982.

Arriza, J. L., W. A. Fairman, J. I. Wadiche, G. H. Murdoch, M. P. Kavanaugh, and S. G. Amara. J. Neurosci. 14:5559-5569, 1994.

Arriza, J. L., M. P. Kavanaugh, W. A. Fairman, Y. N. Wu, G. H. Murdoch, R. A. North, and S. G. Amara. J. Biol. Chem. 268:15329-15332, 1993.

Ash, J. F., R. P. Igo, M. Morgan, and A. Grey. Somat. Cell Mol. Genet. 19:237-243, 1993.

Atwell, D., B. Barbour, and M. Szatkowski. Neuron. 11:401-407, 1993.

Bannai, S., H. N. Christensen, J. V. Vadgama, J. C. Ellory, and E. Engelsberg. Nature. 311:308-310, 1984.

Bannai, S. and T. Ishii. J. Cell Physiol. 137:360-367, 1988.

Barber, E. F., M. E. Handlogten, and M. S. Kilberg. J. Biol. Chem. 258:1185-1191, 1983.

Barber, E. F., M. E. Handlogten, T. A. Vida, and M. S. Kilberg. J. Biol. Chem. 257:14960-14966, 1982.

Barker, D. J. P. Horm. Res. 42:223-230, 1994.

Battaglia, F. C. J. Anim. Sci. 70:3258-3263, 1992.

Bernardini, I., M. I. Evans, K. H. Nicolaides, D. L. Economides, AND W. A. Gahl. Am. J. Obstet. Gynecol. 164:481-1490, 1991.

Bertran, J., A. Werner, J. Chillaron, V. Nunes, J. Biber, X. Testar, A. Zorzano, X. Estivill, H. Murer, and M. Palacin. J. Biol. Chem. 268:14842-14849, 1993.

Bertran, J., A. Werner, M. L. Moore, G. Stange, D. Markovich, J. Biber, X. Testar, A. Zorzano, M. Palacin, And H. Murer. Proc. Natl. Acad. Sci. 89:5601-5605, 1992.

Bertran, J., A. Werner, G. Stange, D. Markovich, J. Biber, X. Testar, A. Zorzano, M. Palacin, and H. Murer. Biochem. J. 281:717-723, 1992

Blakely, R. D., J. A. Clark, T. Pacholczyk, and S. G. Amara. J. Neurochem. 56:860-871, 1991.

Bonini, J. A. and C. Hofmann. Biotechniques 11:708-709, 1991.

Booth, A. G., R. O. Olaniyan, and O. A. Vanderpuye. Placenta 1: 327-336, 1980.

Booth, A. G., and O. A. Vanderpuye. CIBA Found. Symp. 95:180-194, 1983.

Borke, J. L., A. Caride, A. K. Verma, L. K. Kelley, C. H. Smith, J. T. Penniston, and R. Kumar. Am. J. Physiol. 257:C341-C346, 1989.

Bowers, G. N., Jr. and R. B. McComb. Clin. Chem. 12: 79-80, 1966.

Boyd, C. A. R., A. R. Chipperfield, and L. A. Steele. Placenta 1: 361-377, 1979.

Bracero, L. A., D. Beneck, N. Kirshenbaum, M. Peiffer, P. Stalter, and H. Schulman. Am. J. Obstet. Gynecol. 161: 388-393, 1989.

Bracy, D. S., M. A. Schenerman, and M. S. Kilberg. Biochim. Biophys. Acta 899:51-57, 1987.

Bravo, P., M. Y. el Mir, M. A. Serrano, R. Boyd, and J. J. Marin. Am. J. Physiol. 265:G242-G250, 1993.

Busch, A. E., T. Herzer, S. Waldegger, F. Schmidt, M. Palacin, J. Biber, D. Markovich, H. Murer, and F. Lang. J. Biol. Chem. 269:25581-25586, 1994

Bussolati, O., P. C. Laris, F. A. Nucci, V. Dall'Asta, N. Longo, G. G. Guidotti, and G. C. Gazzola. Am. J. Physiol. 253:C1-C7, 1987.

Calonge, M. J., P. Gasparini, J. Chillaron, M. Chillon, M. Gallucci, F. Rousaud, L. Zelante, X. Testar, B. Dallapiccola, F. Di Silverio, P. Barcelo, X. Estivill, A. Zorzano, V. Nunes, and M. Palacin. Nature Genetics. 6:420-425, 1994.

Campa, M. J. and M. S. Kilberg. J. Cell. Physiol. 141:645-652, 1991.

Campione, A. L., and L. J. Van Winkle. Biol. Reprod. 50 (Suppl):71A, 1994.

Cariappa, R. and M. S. Kilberg. Am. J. Physiol. In Press: 1992.

Casado, M., A. Bendahan, F. Zafra, N. C. Danbolt, C. Aragon, C. Gimenez, B. I. Kanner. J. Biol. Chem. 268:27313-27317, 1993.

Cassano, G., B. Leszczynska, and H. Murer. Pflugers Arch. 397:114-120, 1983.

Cetin, I., P. V. Fennessey, A. N. Quick, A. M. Marconi, G. Meschia, F. C. Battaglia, and J. W. Sparks. Am. J. Physiol. 260:E371-E378, 1991.

Cetin, I., P. V. Fennessey, J. W. Sparks, G. Meschia, and F. C. Battaglia. Am. J. Physiol. 263:E786-E793, 1993.

Cetin, I., A. M. Marconi, C. Corbetta, A. Lanfranchi, A. M. Baggiani, F. C. Battaglia, and G. Pardi. Early Hum. Dev. 29: 183-186, 1992.

Chomczynski, P. and N. Sacchi. Anal. Biochem. 162:156-159, 1987.

Christensen, H. N. J. Biol. Chem. 239:3584-3589, 1964.

Christensen, H. N. Physiol. Rev. 62:1193-1195, 1982.

Christensen, H. N. in Biological Transport. W. A. Benjamin Press, Reading, MA, 1987.

Christensen, H. N. and M. E. Handlogten. FEBS Lett. 3:14-17, 1969a.

Christensen, H. N., M. E. Handlogten, I. Lam, H. S. Tager, and R. Zand. J. Biol. Chem. 244:1510-1520, 1969.

Christensen, H. N., M. E. Handlogten, and E. L. Thomas. Proc. Natl. Acad. Sci. 63:948-955, 1969c.

Christensen, H. N., M. S. Kilberg, and M. Handlogten. J. Biol. Chem. 266:2344-2349, 1982b.

Christensen, H. N. and M. S. Kilberg. in Amino Acid Transport in Animal Cells. Manchester Univ. Press, Manchester, England pp. 10-46, 1987.

Christensen, H. N. and M. Liang. J. Biol. Chem. 241:5542-5551, 1966.

Christensen, H. N., M. Liang, E. G. Archer. J. Biol. Chem. 242:5237-5246, 1967.

Christensen, H. N., D. L. Oxender, M. Liang, and K. A. Katz. J. Biol. Chem. 240:3609-3616, 1965.

Christensen, H. N. and J. A. Streicher. J. Biol. Chem. 175:95-100, 1948.

Closs, E. I., L. M. Albritton, J. W. Kim, and J. M. Cunningham. J. Biol. Chem. 268:7538-7544, 1993a.

Closs, E. I., C. R. Lyons, C. Kelly, and J. M. Cunningham. J. Biol. Chem. 268:20796-20800, 1993b.

Closs, E. I., I. H. Rinkes, A. Bader, M. L. Yarmush, and J. M. Cunningham. J. Virology 67:2029-2102, 1993.

Coady, M. J., F. Jalal, X. Chen, G. Lemay, A. Berteloot, and J. Y. Lapointe. FEBS Lett. 356:174-178, 1994

Collarini, E. J., and D. L. Oxender. Ann. Rev. Nutr. 7:75-87, 1987.

Colombo, J. P., H. Cervantes, M. Kokorovic, V. Pfister, and R. Perritaz. Ann. Nutr. Metab. 36:23-33, 1992.

Conradt, M., T. Storck, and W. Stoffel. Eur. J. Biochem. 229:682-687, 1995

Coons, A. H. and M. H. Kaplan. J. Exp. Med. 91:1-13, 1950.

Danbolt, N. C., G. Pines, B. I. Kanner. Biochemistry 29:6734-6740, 1990.

Danbolt, N. C., J. Storm-Mathisen, and B. I. Kanner. Neuroscience 51:295-310, 1992.

Dantzig, A. H., C. W. Slayman, and E. A. Adelberg. Somat. Cell Genet. 8:509-513, 1982.

Devés, R., S. Angelo, and P. Chavez. J. Physiol. 468:753-766, 1993.

Devés, R., P. Chavez, and C. A. R. Boyd. J. Physiol. 454:491-501, 1992.

Dicke, J. M. and G. I. Henderson. Am. J. Med. Sci. 295:223-227, 1988.

Eaton, B. M., and D. L. Yudilevich. Am. J. Physiol. 241:C106-C112, 1981.

Economides, D. L., K. H. Nicolaides, W. A. Gahl, I. Bernadini, and M. I. Evans. Am. J. Obstet. Gynecol. 161:1219-1227, 1989.

Eisenbarth, G. S., B. F. Haynes, J. A. Schroer, A. S. Fauci. J. Immunol. 124:1237-1244, 1989.

Eisenberg, D., E. Schwartz, M. Komaromy, and M. Wall. J. Mol. Biol. 179:125-142, 1984.

Esko, J. D. Meth. Cell Biol. 32:387-392, 1989.

Fairman, W. A., R. J. Vandenberg, J. L. Arriza, M. P. Kavanaugh, and S. G. Amara. Nature. 375:599-603, 1995.

Fei, Y. J., P. D. Prassad, F. H. Leibach, and V. Ganapathy. Biochemistry. 34:8744-8751, 1995.

Finley, K. D., D. K. Kakuda, A. Barrieux, J. Kleeman, P. D. Huynh, and C. L. MacLeod. Proc. Natl. Acad. Sci. USA, in press, 1995.

Fong, A. D., M. E. Handlogten, and M. S. Kilberg. Biochim. Biophys. Acta 1022:325-332, 1989.

Fremeau, R. T., M. G. Caron, and R. D. Blakely. Neuron. 8:915-926, 1992

Furesz, T. C., A. J. Moe, and C. H. Smith. Am. J. Physiol. 261:C246-C252, 1991.

Furesz, T. C., A. J. Moe, and C. H. Smith. Am. J. Physiol. 268:C755-C761, 1995.

Furriols, M., J. Chillaron, C. Mora, A. Castello, J. Bertran, M. Camps, X. Testar, S. Vilaro, A. Zorzano, and M. Palacin. J. Biol. Chem. 268:27060-27068, 1993.

Ganapathy, M. E., F. H. Leibach, V. B. Mabesh, L. D. Devoe, and V. Ganapathy. Biochem J. 238:201-208, 1986.

Garcia-Sancho, J., A. Sanchez, and H. N. Christensen. Biochim. Biophys. Acta. 464:295-312, 1977.

Gazzola, G. C., V. Dall'Asta, O. Bussolati, M. Makowske, AND H. N. Christensen. J. Biol. Chem. 256: 6054-6059, 1981.

Gebhardt, R., and R. Mecke. EMBO J. 2:557-567, 1983.

Glazier, J. D., C. J. P. Jones, and C. P. Sibley. Placenta 11: 451-163, 1993.

Golde, S. H. in Intrauterine Growth Retardation. Year Book Medical Publishers. Chicago, IL. pp. 3-4, 1989.

Gomezangelats, M., B. Ruizmontasell, A. Felipe, J. J. G. Marin, F. J. Casado, and M. Pastoranglada. Am. J. Physiol. 31: E368-E374, 1995.

Greenberg, M. E. and E. B. Ziff. Nature 311:433-438, 1984.

Greizerstein, H. B. Biol. Reprod. 26:847-853, 1980.

Gresham, E., E. James, J. Raye, F. C.Battaglia, E. Makowske, and G. Meschia. Pediatrics 50:372-379, 1972.

Guastella, J., N. Nelson, H. Nelson, L. Czyzyk, S. Keynan, M. C. Miedel, N. Davidson, H. A. Lester, and B. I. Kanner. Science 249:1303-1306, 1990.

Guidotti, G. G. and G. C. Gazzola. in Mammalian Amino Acid Transport. Plenum Press. New York, NY, 1992.

Handlogten, M. E. and M. S. Kilberg. J. Biol. Chem. 259:3519-3525, 1984.

Harvey, C. M., W. R. Muzyka, S. Y. M. Yao, C. I. Cheeseman, and J. D. Young. Am. J. Physiol. 265:G99-G106, 1993.

Haüssinger, D., in Hepatic Transport of Organic Substances. Springer-Verlag. Berlin, Germany. pp 157-166, 1989a.

Haüssinger, D., B. Stoll, T. Stehle, and J. P. Colombo. Biochem. J. 264:837-843, 1989b.

Hediger, M. A. J. Exp. Biol. 196:15-49, 1995.

Hediger, M. A., M. J. Coady, T. S. Ikeda, and E. M. Wright. Nature 330:379-381, 1987.

Hees, B., N. C. Danbolt, B. I. Kanner, W. Hasse, K. Heitmann, and H. Koepsell. J. Biol. Chem. 267:23275-23281, 1992.

Heitmann, R. N., and E. N. Bergman. Am. J. Physiol. 241:E465-E472, 1981.

Hemler, M. E., and J. L. Strominger. J. Immunol. 129:623-628, 1982.

Hems, R., M. Stubbs, and H. A. Krebbs. Biochem. J. 107:807-815, 1968.

Hirono, C., S. Yamagishi, R. O'Hara, Y. Hisanga, T. Nakayama, and H. Sugiyama. Brain Res. 359:57-64, 1983.

Hoeltzli, S. D., L. K. Kelley, A. J. Moe, and C. H. Smith. Am. J. Physiol. 259: C47-C55, 1990.

Hoeltzli, S. D., and C. H. Smith. Am. J. Physiol. 256:C630-C637, 1989.

Hofmann, K., M. Duker, T. Fink, P. Lichter, and W. Stoffel. Genomics. 24:20-26, 1994.

Holzman, I. R., J. A. Lemmons, G. Meschia, and F. C. Battaglia. Proc. Soc. Exp. Biol. Med. 156:27-30, 1977.

Iioka, H. I., I. Moriyama, K. Itoh, K. Hino, and M. Ichija. J. Obstet. Gynaecol. Jpn. 37: 2005-2009, 1985.

Jaenisch, R. and E. Hoffman. Virology 98:289-297, 1979.

Jansson, T., M. Wennergren, and N. P. Illsley. J. Clin. Endocrinol. Metab. 77: 1554-1562, 1993.

Johnson, L. W., and C. H. Smith. Am. J. Physiol. 254: C773-C780, 1988.

Jungerman, K., and N. Katz. Physiol. Rev. 69:708-764, 1989.

Kakuda, D. K., K. Finley, V. E. Dionne, and C. L. MacLeod. Transgene 1:91-101, 1993.

Kanai, Y., and M. A. Hediger. Nature 360:467-471, 1992.

Kanai, Y., S. Nussberger, M. F. Romero, W.F. Boron, S. C. Hebert and M. A. Hediger. J. Biol. Chem. 270:16561-16568, 1995 .

Kanai, Y., C. P. Smith, and M. A. Hediger. FASEB J. 8:1450-1459, 1994.

Kanai, Y., M. G. Stelzner, W. Lee, R. G. Wells, D. Brown, and M. A. Hediger. Am. J. Physiol. 263:F1087-F1093, 1992.

Karl, P. I., and S. E. Fisher. Alochol. Clin. Exp. Res. 18: 942-946, 1994.

Karl, P. I., H. Tkaczevski, and S. E. Fisher. Pediatr. Res. 25:19-26, 1989.

Kavanaugh, M. P. Biochemistry 32:5781-5785, 1993.

Kavanaugh, M. P., H. Wang, Z. Zhang, W. Zhang, Y-N. Wu, E. Deschant, R. North, and D. Kabat. J. Biol. Chem. 269:15445-15450, 1994.

Kawakami, H., K. Tanaka, T. Nakayama, K. Inoue, and S. Nakamura. Biochem. Biophys. Res. Comm. 199:171-176, 1994.

Keeffe, E. B., B. F. Scharschmidt, N. M. Blankenship, and R. K. Ockner. J. Clin. Invest. 64:1590-1598, 1979.

Kelley, L. K., C. H. Smith, and B. F. King. Biochim. Biophys. Acta 734: 91-98, 1983.

Kilberg, M. S. TIBS 11:183-186, 1986.

Kilberg, M. S. Methods In Enzymology 173:564-575, 1989.

Kilberg, M. S., E. F. Barber, and M. E. Handlogten. Curr. Topics Cell Regul. 25:133-137, 1985.

Kilberg, M. S., R. G. Hutson, and R. O. Laine. FASEB J. 8: 13-19, 1994.

Kilberg, M. S., B. R. Stevens, and D. A. Novak. Annu. Rev. Nutr. 13:137-165, 1993.

Kilberg, M. S., B. K. Tamarappoo, S. Shafqat, and R. T. Fremeau. in Transport in the Liver. Lancaster, U.K. Kluwer Publ., 1994.

Kim, J. W., E. I. Closs, L. M. Albritton, and J. M. Cunningham. Nature 352:725-728, 1991.

King, B. F. J. Ultrastruct. Res. 85:320-328, 1983.

Kirschner, M. A., N. G. Copeland, D. J. Gilbert, N. A. Jenkins, and S. G. Amara. Genomics 24:218-224, 1994.

Kletzien, R. F., M. W. Pariza, J. E. Becker, and V. R. Potter. Anal. Biochem. 68:537-544, 1975.

Kliman, H. J., J. E. Nestler, E. Sermasi, J. M. Sanger, and J. F. Strauss. Endocrinology 118:1567-1582, 1986.

Kudo, Y. AND C. A. R. Boyd Biochim. Biophys. Acta 1021:169-174, 1990.

Kudo, Y., K. Yamada, A. Fujiwara, and T. Kawasaki. Biochim. Biophys. Acta 904:309-318, 1987.

Laine, R. O., P. J. Laipis, N. F. Shay, and M. S. Kilberg. J. Biol. Chem. 266:16969-16972, 1991.

Lee, W. S., R. G. Wells, R. V. Sabbag, T.K. Mohandas, and M. A. Hediger. J. Clin. Invest. 91:1959-1963, 1993.

Lehre, K. P., L. M. Levy, O. P. Ottersen, J. Storm-Mathisen, and N. C. Danbolt. 1995. J. Neurosci. 15:1835-1853, 1993.

Lemmons, J. A., E. W. Adcock, M. D. Jones, M. A. Naughton, G. Meschia, and F. C. Battaglia. J. Clin. Invest. 58:1428-1434, 1976.

Leoni, S., S. Spagnuolo, L. Dini, and L. C. Devirgilis J. Cell. Physiol. 130:103-110, 1987.

Lever, J. E. CRC Crit. Rev. 7:187-245, 1980.

Levitsky, L. L., B. S. Stonestreet, K. Mink, and Q. Zheng. Am. J. Physiol. 265:E722-E727, 1993.

Lindros, K. E. and K. E. Penttilia. Biochem. J. 228:757-760, 1985.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. J. Biol. Chem. 1983:265-275, 1951.

MacLeod, C. L., K. D. Finley, and D. Kakuda. J. Exp. Biol. 196:109-121, 1994.

MacLeod, C. L., K. Finley, D. Kakuda, C. A. Kozak, and M. F. Wilkinson. Mol. Cell Biol. 10:3663-3674, 1990b.

MacLeod, C. L., A. M. Fong, B. S. Seal, L. Walls, and M. F. Wilkinson. Cell Growth and Differentiation 1:271-279, 1990a.

Magagnin, S., J. Bertran, A. Werner, D. Markovich, J. Biber, M. Palacin, and H. Murer. J. Biol. Chem. 267:15384-15390, 1992

Mahendran, D., P. Donnai, J. D. Glazier, S. W. D'Souza, R. D. H. Boyd, and C. P. Sibley. Pediatr. Res. 34: 661-665, 1993.

Makowske, M., and H. N. Christensen Christensen. J. Biol. Chem. 257:14635-14638, 1982.

Malandro, M. S., M. J. Beveridge, M. S. Kilberg, and D. A. Novak. Am. J. Physiol. 267:C804-C811, 1994.

Manfras, B. J., W. A. Rudert, W. A. Trucco, and B. O. Boehm. Biochim. Biophys. Acta 1195:185-188, 1994.

Marconi, A. M., F. C. Battaglia, G. Meschia, and J. W. Sparks. Am. J. Physiol. 257:E909-E915, 1989.

Margolskee, R. F., P. Kavathas, and P. Berg. Mol. Cell Biol. 8:2837-2843, 1988.

Marin, J. J., M. A. Serrano, M. Y. el Mir, N. Eleno, and C. A. R. Boyd. Gastroenterology 99:1431-1438, 1990.

Markovich, D., G. Stange, J. Bertran, M. Palacin, A. Werner, J. Biber, and H. Murer. J. Biol. Chem. 268:1362-1367, 1993.

Mestyan, J. and G. Soltez. Maternal, fetal, and neonatal amino acid metabolism. in Fetal physiology and medicine. London: Butterworths. pp 177-211, 1984.

Metz, J., A. Aoki, and W. G. Forssman. Cell Tissue Res. 192:391-407, 1978.

Moe, A. J. and C. H. Smith. Am. J. Physiol. 257: C1005-C1011, 1989.

Moores, R. R., P. R. Vaughn, F. C. Battaglia, R. V. Fennessey, R. B. Wilkenberg, and G. Meschia. Am. J. Physiol. 267:R89-R96, 1994.

Mosckovitz, R., E. Heimer, A. Felix, S. S. Tate, and S. Udenfriend. Proc. Natl. Acad. Sci. USA 90:4022-4026, 1993.

Monro, H. N. Fed. Proc. 45:2500-2501, 1986.

Naeye, R. L., W. Blanc, and C. Paul. Pediatrics 52:494-504, 1973.

Nirenberg, M. J., S. S. Tate, R. Mosckovitz, S. Udenfriend, and V. M. Pickel. J. Comp. Neuro. 356:505-522, 1995

Niven, R.W., and H. Schreier. Pharm. Res. 7:1127-1133, 1990.

Novak, D. A., M. J. Beveridge, and M. S. Malandro. Placenta, in press.

Novak, D. A., F. C. Ryckman, and F. J. Suchy. Hepatology 10:447-453, 1989.

Ogata, E. S., M. E. Bussey, A. Labarabera, and S. Finley. Pediatr. Res. 19:32-37, 1985.

Otori, Y., S. Shimada, K. Tanaka, I. Ishimoto, Y. Tano, and M. Tohyama. Mol. Brain Res. 27:310-314, 1994.

Palacin, M. J. Exp. Biol. 196:123-137, 1994.

Pan, M., M. S. Malandro, and B. R. Stevens. Am. J. Physiol. 268:G578-G585, 1995.

Pariza, M. W., F. R. Butcher, R. F. Kletzien, J. E. Becker, and V. R. Potter. Proc. Natl. Acad. Sci. 73:4511-4515, 1976.

Parmacek, M. S., B. A. Karpinski, K. M. Gottesdiener, C. B. Thompson, and J. M. Leiden. Nucl. Acids Res. 17:1915-1931, 1989.

Phillips, A. F., I. R. Holzman, C. Teng, and F. C. Battaglia. Am. J. Obstet. Gynecol. 131:881-887, 1978.

Pickel, V. M., M. J. Nirenberg, J. Chan, R. Mosckovitz, S. Udenfriend, and S. S. Tate. Proc. Natl. Acad. Sci. USA 90:7779-7783, 1993.

Pines, G., N. C. Danbolt, M. Bjoras, Y. Zhang, A. Bendahan, L. Eide, H. Koepsell, J. Storm-Mathisen, E. Seeberg, and B. I. Kanner. Nature 360:464-467, 1992.

Pines, G., Y. Zhang, and B. I. Kanner. J. Biol. Chem. 270:17093-17097, 1995

Plakidou-Dymock, S., and J. D. McGivan. Biochem. J. 295:749-755, 1993.

Pras, E., N. Arber, I. Aksentijevich, G. Katz, J. M. Schapiro, L. Prosen, L. Gruberg, D. Harel, U. Liberman, J. Weissenbach, M. Pras, and D. L. Kastner. Nature Genetics. 6:425-426, 1994.

Puppi, M., and S. J. Henning. Proc. Soc. Exp. Biol. Med. 209:38-45, 1995.

Quackenbush, E., M. Clabby, K. M. Gottesdiener, N. Jones, J. Strominger, S. Speck, and J. M. Leiden. Proc. Natl. Acad. Sci. USA. 84:6526-6530, 1995.

Rauen, T.,and B. I. Kanner. Neuroscience Lett. 169:137-140, 1994.

Reizer, J., K. Finley, D. Kakuda, C. L. MacLeod, A. Reizer, and M. H. Saier. Protein Sci. 2:20-30, 1993.

Remesar, X., L. Arola, A. Palou, and M. Alemany. Eur. J. Biochem. 110:289-293, 1980.

Rennie, M. J., A. Ahmed, S. Y. Low, H. S. Hundal, P. W. Watt, P. A. MacLennan, C. J. Egan, and P. M. Taylor. Biochem. Soc. Trans. 18:1140-1142, 1990.

Ross, B. D., R. Hems, and H. A. Krebs. Biochem. J. 102:942-951, 1967.

Rosso, P. Science 187:648-650, 1975.

Rosso, P. J. Nutri. 107:2002-2005, 1977a.

Rosso, P. in Nutritional Disorders of American Women. John Whiley and Sons. New York, NY. pp. 3-25, 1977b.

Rosso, P. Federation Proc. 39:250-254, 1980a.

Rosso, P. and R. Kava. J. Nutri. 110:2350-2354, 1980b.

Saier, M. H., G. A. Daniels, P. Boerner, and J. Lin. J. Memb. Biol. 104:1-6, 1988.

Sambrook, J., E. F. Fritsch, and T. Maniatis. in Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Press. Cold Spring Harbor, NY. 1989.

Sashidharan, P., I. Wittenberg, and A. Plaitakis. Biochim. Biophys. Acta 1191:393-396, 1994.

Sastry, B V., M. A. Horst, and R. J. Nukam. Placenta 10: 345-358, 1989.

Schafer, J. A., and M. L. Watkins. Pflugers Arch. 401:143-151, 1984

Scharschmidt, B. F., E. B. Keeffe, N. M. Blankenship, and R. K. Ockner. J. Lab. Clin. Med. 93:790-799, 1979.

Schenerman, M. A. and M. S. Kilberg. Biochim. Biophys. Acta. 856:428-431, 1986.

Schneider, H., K. H. Mohlen, J. C. Challier, and J. Dancis. Br. J. Obstet. Gynecol. 86:299-306, 1979.

Segal, S., and S. O. Thier. in The Metabolic Basis of Inherited Diseases, p. 2479-2496. New York: McGraw-Hill, 1989.

Shafqat, S., B. K. Tamarappoo, M. S. Kilberg, R. S. Puranam, J. O. McNamara, A. Guadano-Ferraz, and R. T. Fremeau. J. Biol. Chem. 268:15351-15355, 1993

Shay, N. F., H. S. Nick, and M. S. Kilberg. J. Biol. Chem. 265:17844-17848, 1990.

Shotwell, M. A., M. S. Kilberg, and D. L. Oxender. Biochim. Biophys. Acta. 737:267-279, 1983.

Sideri, M., G. de Virgilis, R. Rainoldi, and G. Remotti. Trophoblast Res. 1:15-26, 1983.

Sigel, E. J. Physiol. 386:73-90, 1987.

Sips, H. J., D. A. DeGraff, and K. Van Dam. Eur. J. Biochem. 122:259-264, 1982.

Smeriglio, V. L. in Intrauterine Growth Retardation. Year Book Medical Publishers. Chicago, IL. pp. 34-53, 1989.

Smit, N. P. M., C. W. T. van Roermund, H. M. F. Aerts, J. C. Jeikoop, M. Vandenberg, S. Pavel, and R. J. A. Waters. Biochim. Biophys. Acta. 1181:1-6, 1993.

Smith, K. E., L. A. Borden, P. R. Hartig, T. A. Branchek, and R. L. Weinshank. Neuron 8:927-935, 1992.

Smith, C. H., A. J. Moe, and V. Ganapathy. Ann. Rev. Nutr. 12:183-206, 1992a.

Smith, C. H., D. M. Nelson, B. F. King, T.M. Donahue, S. Ruzycki, and L. Kelley. Am. J. Obstet. Gynecol. 128:190-196, 1977.

Smith, C. P., S. Weremowicz, Y. Kanai, M. Stelzner, M. A. Hediger, and C. C. Morton. Genomics. 20:335-336, 1994.

Souba, W. W. J. Parent. Enteral. Nutr. 11:569-579, 1987.

Stegink, L. D., R. M. Pitkin, W. A. Reynolds, L. J. Filer, D. P. Boaz, and M. C. Brummel. Am. J. Obstet. Gynecol. 122:70-78, 1975.

Stevens, B. R., J. D. Kauntiz, and E. M. Wright. Ann. Rev. Physiol. 46:417-433, 1984.

Stoll, B., S. McNelley, H-P. Buscher, and D. Haussinger. Hepatology 13:247-253, 1991.

Storck, T., S. Schulte, K. Hoffmann, and W. Stoffel. Proc. Natl. Acad. Sci USA 89:10955-10959, 1992.

Sturman, J. A. J. Nutr. 118:1169-1176, 1988.

Swendseid, M. E., J. Villalobos, and B. Friedrich. J. Nutr. 80: 99-102, 1963.

Swirski, R. A., D. Van den Berg, A. Murphy, C. M. Lambert, E. C. Friedberg, and R. T. Schimke. METHODS: A Companion to Methods in Enzomology 4:133-142, 1992.

Tamarappoo, B. K., K. K. McDonald, M. S. Kilberg. Biochim. Biophys. Acta., in press, 1996.

Tarnuzzer, R. W., M. J. Campa, N. X. Qian, E. Englesberg, and M. S. Kilberg. J. Biol. Chem. 265:13914-13917, 1990.

Tate, S. S., R, Urade, T. V. Getchell, and S. Udenfriend. Arch. Biochem. Biophys. 275:591-596, 1989

Tate, S. S., N. Yan, and S. Udenfriend. Proc. Natl. Acad. Sci. 89:1-5, 1992.

Teixeira, S., S. DiGrandi, and L. C. Kuhn. J. Biol. Chem. 262:9574-9580, 1987.

Tolner, B., B. Poolman, B. Wallace, and W. N. Konings. J. Bacteriol. 174:2391-2393, 1992.

Tse, C. M., D. A. Fincham, J. C. Ellory, and J. D. Young. Biochem. J. 277:565-568, 1991.

Vadgama, J. V., and H. N. Christensen. J. Biol. Chem. 259:3648-3652, 1984.

Van Slyke, D. D. and G. M. Meyer. J. Biol. Chem. 16:197-212, 1913-1914.

Van Vleen, L. C. P., C. Teng, W. W. Hay, G. Meschia, and F. C. Battaglia. Metabolism. 36:48-53, 1987.

Van Winkle, L. J. Biochim. Biophys. Acta. 947:173-208, 1988

Van Winkle, L. J., A. L. Campione, and J. M. Gorman. J. Biol. Chem. 263:3150-3163, 1988.

Van Winkle, L. J., H. N. Christensen, and A. L. Campione. J. Biol. Chem. 260:12118-12123, 1985.

Vandenberg, R. J., J. L. Arriza, S. G. Amara, and M. P. Kavanaugh, M. P. J. Biol. Chem. 270:17668-17671, 1995.

Varma, D. R., and R. Ramakrishnan. Placenta 12: 277-284, 1991.

Vaughn, P. R., C. Lobo, F. C. Battaglia, P. V. Fennessey, R. B. Wilkening, and G. Meschia. Am. J. Physiol. 268:E705-711, 1995.

Velaz-Faircloth, M., T. S. McGraw, M. S. Malandro, R. T. Fremeau, M. S. Kilberg, and K. J. Anderson, Am. J. Physiol., in press.

Voelkl, H., and S. Silbernagl. Pflugers Arch. 359:190-195, 1982

Wang, H., M. P. Kavanaugh, and D. Kabat. Virology 202:1058-1060, 1994.

Wang, H., M. P. Kavanaugh, R. A. North, and D. Kabat. Nature 352:729-731, 1991.

Wang, Y., and S. S. Tate. FEBS Lett. 368:389-392, 1995.

Weissbach, L., M. E. Handlogten, H. N. Christensen, and M. S. Kilberg. J. Biol. Chem. 257:12006-12011, 1982.

Wells, R. G. and M. A. Hediger. Proc. Natl. Acad. Sci. 89:5596-5600, 1992.

Wells, R. G., A. M. Pajor, Y. Kanai, E. Turk, E. M. Wright, and M. A. Hediger. Am. J. Physiol. 263:F459-F465, 1992.

Wheeler, C. P. D. and D. L. Yudilevich. Biochim. Biophys. Acta 978:257-266, 1989.

White, M. F. Biochim. Biophys. Acta 822:355-374, 1985.

White, M. F. and H. N. Christensen. J. Biol. Chem. 257:4450-4457, 1982.

White, M. F., G. C. Gazzola, and H. N. Christensen. J. Biol. Chem. 257:4443-4449, 1982.

Wolfram, S., H. Giering, and E. Scharrer. Comp. Biochem. Physiol. 78A:475-480, 1984.

Woodward, M. H., W. A. Dunn, R. O. Laine, M. Malandro, R. McMahon, O. Simell, E. R. Block, and M.S. Kilberg. Am. J. Physiol. 266:E817-E824, 1994.

Wu, J. Y., D. Robinson, H. Kung, and M. Hatzoglou. J. Virology. 68:1615-1623, 1994

Yamaguchi, R., T. Matsuda, and T. Aizawa. Tohoku J. Exp. Med. 125:367-376, 1978.

Yan, N., R. Mosckovitz, L. D. Gerber, S. Mathew, V. V. Murty, S. S. Tate, and S. Udenfriend. Proc. Natl. Acad. Sci. USA 91:7548-7552, 1994.

Yan, N., R. Mosckovitz, S. Udenfriend, S. S. Tate. Proc. Natl. Acad. Sci. USA 89:9982-9985, 1992.

Yoshimoto, T., E. Yoshimoto, and D. Meruelo. Virology 185:10-17, 1991.

Young, M. in Placental Transfer. Pitman Medical Publishing Co. Kent,England. pp. 142-158, 1979.

Young, M. AND E. M. Widdowson. Biol. Neonate 27:184-191, 1975.

Yudilevich, D. L., and J. H. Sweiry. Biochim. Biophys. Acta 822:169-201, 1985.

Zerangue, N., Arriza, J. L, S. G. Amara, and M. P. Kavanaugh, J. Biol. Chem. 270:6433-6435, 1995.

Zhang, Y., G. Pines, and B. I. Kanner. J. Biol. Chem. 269:19573-19577, 1994

BIOGRAPHICAL SKETCH

Marc Shane Malandro was born to Eugene and Jean Malandro on April 27, 1966 in Brookfield, Ohio. He attended Brookfield High School and subsequently earned both a B.S. and M.S. in Biological Sciences from Youngstown State University in Youngstown, Ohio. Marc began in the Department of Biochemistry and Molecular Biology at the University of Florida in August of 1991. After graduation, he will begin a postdoctoral fellowship at Case Western Reserve University in Cleveland, Ohio under the direction of Dr. Rob Nicholls. Marc is married to Jennifer Malandro, and they have one daughter, Nicole.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Michael S Kilberg

Michael S. Kilberg, Chair
Professor of Biochemistry
and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Donald A. Novak
Associate Professor of
Pediatrics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Brian D Cain

Brian D. Cain
Associate Professor of
Biochemistry and
Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Susan C. Frost

Susan C. Frost
Associate Professor of
Biochemistry and
Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Harry S. Nick

Harry S. Nick
Associate Professor of
Biochemistry and
Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Bruce R Stevens

Bruce R. Stevens
Associate Professor of
Physiology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1995

Mohan K Raizada

Dean, College of Medicine

Karen A. Holbrook

Dean, Graduate School

UNIVERSITY OF FLORIDA
3 1262 08554 3436